HUMAN FOLLICLE-STIMULATING HORMONE

BY

PAUL ROOS

Uppsala
1967

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HUMAN FOLLICLE-STIMULATING HORMONE

Its isolation from the pituitary gland and from postmenopausal urine and a study of some chemical, physical, immunological, and biological properties of the hormone from these two sources

BY

PAUL ROOS

Almqvist & Wiksells
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PREFACE

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Paul Roos

I. INTRODUCTION

In all craniate vertebrates there is present underneath the brain a gland called the *hypophysis* or the *pituitary*. The first name, the hypophysis, refers directly to the position of the gland in relation to the brain (from the Greek *hypo* – under and *physis* – nature, origin). The second and most commonly used name, the pituitary (from the Latin *pituita* – phlegm, mucus), emerges from the erroneous idea of the gland being the producer of the mucus of the nose.

Anatomically and physiologically the pituitary is divided into two main lobes, the anterior lobe and the posterior lobe. In most species there is also a more or less discrete third lobe, the intermediate lobe. The pituitary belongs to the endocrine or ductless glands. It is characteristic of an endocrine gland that it secretes one or more specific substances, *hormones*, directly into the blood stream: these are conveyed to other parts of the body and giving rise there to changes in the physiological course. As to the chemical composition of hormones, a variety of different types of substances are represented. The known hormones of pituitary origin are peptides or proteins (simple or conjugated).

The mode of action of a hormone is manifested either by an excitation or by an inhibition of physiological processes (an excitation is implicit already in the designation hormone, from the Greek *hormon*, present participle of *hormaein* – to excite). A hormone can exert its action on a group of cells belonging to a specific target organ or/and on an individual cell which cannot be ascribed to a specific tissue. It should be emphasized, however, that the directly measurable effect of a hormone should be considered as a series of successive events. The study of the immediate hormonal effect at the molecular level is a branch of endocrinology as yet only in its initial stages. One factor strongly limiting work along these lines has been the lack of pure hormones. The search for the receptor mechanisms at subcellular levels will no doubt be extensively pursued within the near future.

Current hypotheses and theories suggest at least three different explanations for the mechanism of hormone action. Hormones are considered to act (1) by controlling the activity of specific enzymes, (2) by changing the

permeability of biological membranes or (3) by gene regulation of protein synthesis.

Hormones synthesized in and secreted from the *anterior* pituitary control sexual development and reproduction, bodily growth and other metabolic processes, and thyroid activity, and are also involved in the response of the body to stress and disease. The anterior pituitary exercises the main part of this control by governing the secretion from other endocrine glands and, by virtue of this integrating role is therefore of outstanding importance among the body's endocrine organs.

The function of the anterior pituitary is itself regulated to a large extent by impulses from many structures in the central nervous system. Signals from the midbrain and from the cerebral cortex are transmitted mainly via the nuclei of the hypothalamus to the anterior pituitary. These two organs are connected by the flow of substances termed *neurohumors* which are secreted by the hypothalamus and carried through a special system of blood vessels (portal veins) to the anterior pituitary. The neurohumors (or releasing-factors) promote the synthesis and release of hormones from the pituitary tissue in a hitherto unknown manner. The circuit is closed and balance in the hypothalamo-hypophyseal system is created by the feed-back action of the circulating products of hormonal secretions upon the receptor sites in the central nervous system.

Efforts to prepare hormones of anterior pituitary origin in pure form have proceeded for about 40 years. The progress made during the past years is certainly great; if, however, the lengthy struggle of all those involved in this work is considered, the total outcome is small. Pituitaries from domestic animals have mostly been employed as starting material, but during the last decade the domain has been extended to include human pituitaries and, as a source of excreted hormones, also human urine.

There are, of course, many reasons for the discrepancy mentioned above between effort and progress, and it is easy to give more or less speculative explanations. Indisputable facts, however, have been the limited supply of starting material (this is especially the case for human pituitary glands), and the very complex mixture of closely related chemical substances present in the glands and in urine. Other facts are the low levels at which the individual hormones occur, the often unstable nature of protein hormones, and the problem of obtaining biological and immunological assays which are specific, sensitive, accurate and rapid.

Excellent clinical achievements have been attained with hormone preparations from the above two human sources. Pioneering work within this field has been performed by Gemzell *et al.* [1] (treatment of female sterility) and by Raben [2] (treatment of hypo-pituitary dwarfism). These clinical

findings and the failure to reach similar results with animal hormone preparations have focused present-day interest on the isolation and characterization of the human hormones.

There is a vast amount of literature dealing with those aspects of endocrinology which have been outlined here; basic information is provided in references 3–9.

In this thesis, hormones of *human pituitary origin* have been studied. The thesis deals almost exclusively with one of these hormones the *follicle-stimulating hormone* (*FSH*). The target organs for the action of FSH are the sex glands, the *gonads*. FSH is therefore called a *gonadotropic hormone* or a *gonadotropin*.

FSH, which is a glycoprotein, was prepared from two different sources, namely whole pituitary glands and postmenopausal urine. To differentiate between these two preparations the designations pituitary FSH and urinary FSH are employed.

In connection with descriptions of the isolation and characterization of the two forms of FSH, some of the clinical results of the application of a pituitary FSH concentrate in the treatment of male and female sterility are given.

The human pituitary produces one gonadotropin in addition to FSH, namely *luteinizing hormone* (*LH*) or, as it is also called, *interstitial cell-stimulating hormone* (*ICSH*). It is an intricate and still unsettled question, whether FSH can be prepared completely free of LH activity. The motive to include here some recent studies on pituitary LH is that the highly purified LH obtained offers a good reference substance for discussing the content of LH in FSH.

Finally a third protein hormone emerging from the human anterior pituitary will be briefly discussed, namely *growth hormone* (*GH*). This hormone controls the size of the individual and is in some way involved in a variety of metabolic processes in the body. The work on the purification of the gonadotropins provided a side fraction with growth promoting activity. This fraction was further purified and resulted in a GH preparation which has been clinically used. The successful clinical application of the hormone and the simplicity of the method of preparation make this subject worth mentioning.

II. MATERIALS

II:1. Pituitary Material

The source of glands was whole human pituitaries collected at autopsies in hospitals in Sweden and abroad. Depending on the immediate treatment of the pituitaries at the places of collection two different groups or classes of glands have been available. At most hospitals the glands were immediately frozen, stored at below −20°C, and delivered to Uppsala in a frozen condition. At a few hospitals, however, the glands were put into acetone, kept at low temperature (the limit being set by the facilities available) and sent to Uppsala in acetone but without any cooling precautions. Frozen pituitaries were exclusively employed as starting material in those preparations which were intended to give hormones for physical, chemical and immunological studies. For clinical purposes on the other hand, both classes of glands were utilized.

II:2. Urinary Material

The hormone concentrate used in this study was an experimental batch prepared from postmenopausal urine by N.V. Organon, Oss, Holland (unpublished procedure). A total of 10 g of this material was supplied, derived from about 6000 liters of urine.

III. METHODS

III:1. *Purification*

A. Pituitary and Urinary FSH

A:1. Extraction and Ammonium Sulphate Fractionation

a. *Pituitary Material*

a:1. *Whole frozen glands*

The frozen glands were counted in the coldroom and the weight of the batch to be processed was quickly estimated at room temperature using an open-panned Metler balance. In the following all procedures were performed at 0–4°C.

Sodium phosphate buffer (0.03 *M*, pH 5.7) was poured into a graduated cyliner to a volume corresponding to 5 ml per g of pituitary tissue. The glands and the buffer were transferred to a homogenizer (Turmix), which was immediately operated at full speed for 2 min. Extraction of the homogenate for one hour with continuous stirring was followed by centrifugation in a refrigerated centrifuge (Lourdres) for 10 min at 20,000 × *g*. Some material floated on top of the supernatant and was eliminated by filtration through Pyrex glass-wool. The filtrate, dark red on account of the presence of hemoglobin, was temporarily stored in a beaker surrounded by ice-water. In order to collect further extractable material the precipitates from the centrifuge tubes were again transferred to the homogenizer, for a further treatment. One quarter of the buffer volume used in the original extraction was added and the homogenizer was run at full speed for one minute. Centrifugation and filtration were repeated as before and the new filtrate was added to the original. Two more washings were performed; the treatment in the homogenizer was, however, omitted and replaced by dispersion of the residue in a beaker by manual stirring. The volume of buffer used each time was now reduced to one eighth of the volume employed in the first extraction. The last two washings were collected as described above and added to the other filtrates. The pH value of the pooled filtrates was adjusted to 7.0 by a slow addition of 0.1 *N* NaOH and subsequently the total volume was measured. This solution will hereafter be denoted as extract A.

Extract A was mixed with continuous stirring with an equal volume of saturated ammonium sulphate solution slowly added from a separatory funnel. (The ammonium sulphate solution was equilibrated at 4°C. It

was prepared by dissolving 760 g $(NH_4)_2SO_4$ per liter of boiling distilled water and was kept at 4°C for at least a fortnight prior to use.) The stirring was continued for 1 h after completing the addition and the precipitate which formed, was collected by centrifugation in the same manner as before. The precipitate contained all the growth hormone activity extractable from the pituitaries under the conditions used. It was suspended in distilled water, dialyzed and either lyophilized or stored at −20°C. The precipitate was used as starting material for the preparation of growth hormone as described by Roos *et al.* [10].

To the supernatant (called extract B) were added succesively and with stirring 175 g solid ammonium sulphate per liter solution. After 1 h the two phases which had formed were separated by centrifugation as above. The supernatant (extract C) was discarded; the precipitate was dissolved by the addition of distilled water and the dark red solution was immediately dialyzed. It contained virtually all the gonadotropic activity extracted from the glands. If the hormone concentrate was intended as starting material for purification of FSH and LH, the dialysis was performed against 0.02 *M* potassium phosphate buffer, pH 7.0. If, on the other hand, the aim was a preparation for clinical purposes, the concentrate was the final product and was dialyzed against 0.001 *M* ammonium bicarbonate buffer and then lyophilized.

a:2. *Acetone-preserved glands*

The technique used for work with acetone-preserved glands did not deviate in principle from that discussed above for frozen glands.

The glands were minced in the homogenizer in pre-chilled acetone at −20°C. The mince was freed from most of the acetone by filtration with suction, resuspended in fresh acetone and filtered again. For a further removal of acetone the homogenate was spread out on big sheets of filter paper and left for 15 min at room temperature. It was then suspended in distilled water and lyophilized. The extraction (3 ml buffer per gland) and ammonium sulphate fractionation were performed as described for fresh glands. It was found, however, that the amount of solid $(NH_4)_2SO_4$ added per liter of solution had to be increased to 225 g to ensure complete precipitation of FSH. The precipitate was collected by centrifugation, dissolved in water, dialyzed against distilled water, and finally lyophilized.

A:2. DEAE-Cellulose Chromatography

a. *Pituitary Material*

After dialysis the gonadotropin concentrate was fractionated by DEAE-cellulose column chromatography. Prior to use, the adsorbent (Eastman

Organic Chemicals, Rochester 3, N.Y., USA) was successively washed with 0.5 *N* HCl, distilled water, 0.5 *N* NaOH and again with distilled water. The cellulose was left in contact with the strong acid and base for about 5 min and then immediately washed with water in a Büchner funnel. It was then transferred to a beaker and suspended in the starting buffer (0.02 *M* potassium phosphate, pH 7.0) and allowed to settle. The supernatant, including non-sedimenting fines, was eliminated by suction from the surface. Fresh buffer was added and the procedure was repeated two or three times. The adsorbent was resuspended in fresh buffer and the slurry was poured into the column tube. A column bed of about 200 ml was used for each g of protein to be applied. Starting buffer was passed through the column until the conductivity and pH values of the effluent were identical to those of the buffer. The same comparison between the starting buffer and the hormone concentrate was also performed before the chromatographic run was started. Elutions were carried out at constant pH (7.0) by stepwise increases in the buffer concentration. The flow rate was regulated by a Mariotte flask and the distribution of proteins over the various fractions collected, was estimated by spectrophotometric readings at 280 mμ. Pooled or individual fractions were assayed for biological activity. The result of the assay was the basis for the selection of fractions for further purification. These fractions were pooled and concentrated by ultrafiltration [11].

b. *Urinary Material*

Application of DEAE-cellulose chromatography for purification of the urinary concentrate was essentially as described above; the only difference was that a column volume of 300 ml per g of protein was employed.

A:3. Molecular Sieve Chromatography

Further fractionation of the active materials (pituitary and urinary) was obtained by molecular sieve chromatography on Sephadex® G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Only particles of a size between 270 and 300 mesh were utilized. A system of sieves with decreasing pore sizes was used for the separation of these granules from the dry commercial product. The particles were left for swelling (at 4°C) in 0.1 *M* potassium phosphate buffer (pH 7.0) containing 0.3 *M* sodium chloride. After a minimum of 3 days the column was packed. The column tube was filled with buffer and was equipped on top with a funnel and an electric stirrer. The slurry was poured into the funnel and the gel grains were allowed to sediment with continuous stirring. After packing to a height of 2–3 cm the column tube was opened at the bottom, permitting passage of the buffer.

The packing procedure was then terminated at a maximal flow of buffer. To facilitate the application of the starting zone a filter paper was placed on top of the gel. A constant flow rate of 3 to 4 ml/cm²/h was maintained by a Mariotte flask.

A:4. Hydroxylapatite Chromatography

The next step in the purification procedure of the two forms of FSH was adsorption chromatography on columns of hydroxylapatite. The adsorbent was prepared according to Tiselius *et al.* [12] and was available from Bio-Rad Laboratories, Richmond, California, USA (under the trade name Bio-Gel HT).

The adsorbent was repeatedly washed in a beaker with portions of the starting buffer (0.001 *M* potassium phosphate, pH 7.8), the smallest particles being eliminated by decantation. Packing of columns and checking of the attainment of equilibrium were performed as described above (section a, ƒ p. 16 f.). A column volume of 2 ml was packed for each mg of material to be fractionated. Desorption was performed by a stepwise increase of the phosphate concentration at constant pH.

A:5. Polyacrylamide Gel Electrophoresis

The final step in the purification procedure for both pituitary and urinary FSH was preparative zone electrophoresis in polyacrylamide gel. Cross-linked polyacrylamide was introduced as a stabilizing agent by Raymond and Weinstraub [13], Davis and Ornstein [14] and independently by Hjertén [15] who also performed the first preparative runs.

The construction of the electrophoresis apparatus employed in the present study has been outlined previously [16, 17] and will be described here in more detail.

a. *The Electrophoresis Apparatus*

a:1. *Principal parts*

Construction details of the electrophoresis apparatus are shown in Fig. 1 and all designations in the following refer to this figure. The principal parts of the apparatus are the two concentric glass tubes T and C (which confine the homogeneous gel bed G) and the elution chamber E.

The tube T is 30 cm long, with an inner diameter of 2.1 cm. The length and diameter of the cooling tube C are 35 cm and 0.8 cm, respectively. The elution chamber is made of plexiglass and consists of three separate parts, E1, E2 and E3.

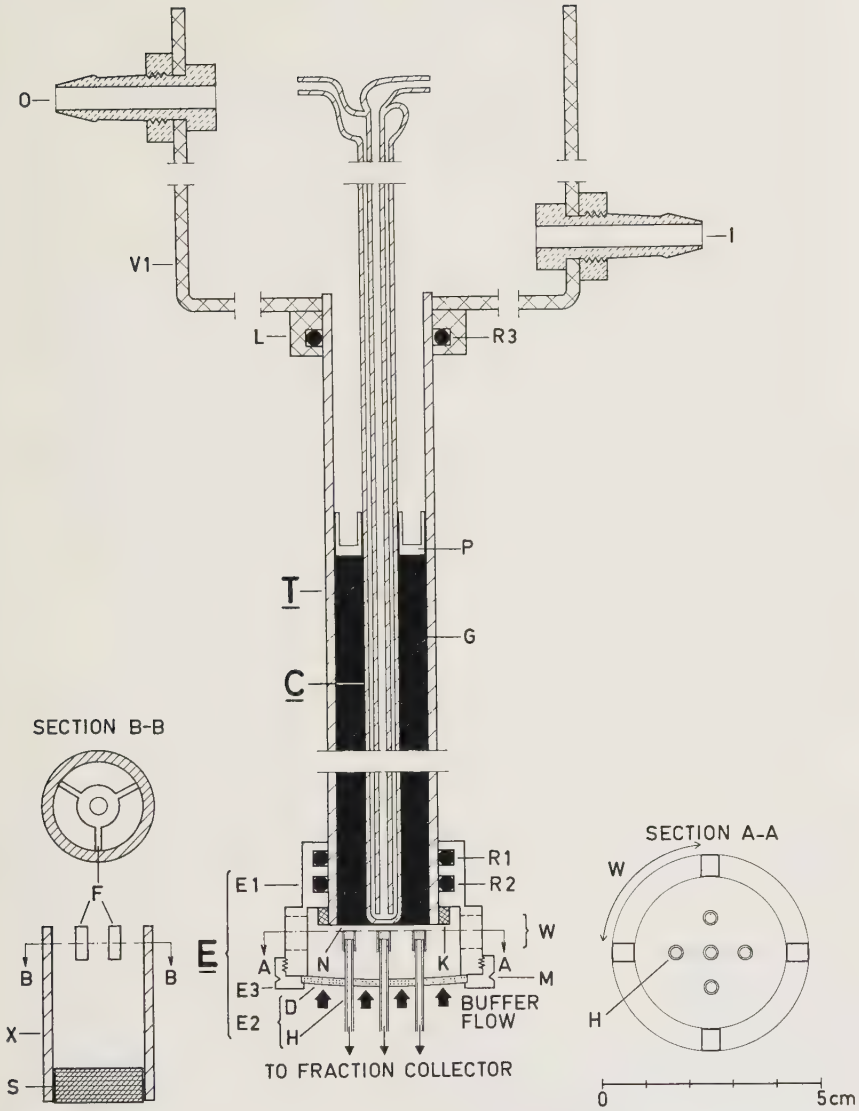

Fig. 1. Apparatus for polyacrylamide gel electrophoresis. The principal parts are the two concentric glass tubes T and C and the elution chamber E. For a detailed description see the text.

Part E1 is equipped with two O-rings, R1 and R2, to ensure a good fit between this part and the tube T. The four symmetrically placed cuttings[1], W, make the construction of part E1 very spacious. (The positions and heights

[1] A construction suggested by Dr S. Hjertén.

of the cuttings are indicated by dotted lines. The width of a cutting is shown in section A–A.)

Part E2 consists of a circular porous disc D, with a diameter bigger than that of the column tube. Also belonging to part E2 are five plastic tubes H inserted by force-fitting through the disc D. One of the tubes is placed in the extension of the axis of the tubes T and C and the other four are symmetrically arranged on the circumference of a circle having a diameter slightly less than that of the tube T. The inner diameter of the tubes H is 0.1 cm. On top of each of them another tube (length 0.5 cm; inner diameter 0.2 cm) is attached by force-fitting and terminated by a porous disc (diameter 0.2 cm). The five tubes are adjusted so that their orifices are in the same horizontal plane and at a level 0.1–0.2 cm below the lower surface of the gel column G. (To prevent the gel from clogging the small porous discs, the column is terminated by a nylon net N (pore size 28 μ). The net is stretched and held in position by a polyethylene ring K pressed on the column tube.)

Part E3 is a supporting ring with a circular track in which part E2 can be force-fitted. The final assembly of the elution chamber is made by screwing the combined parts E2 and E3 on to the part E1.

a:2. *Additional equipment*

During the polymerization process the bottom part of the column tube is extended by the tube X (length 4 cm, inner diameter equal to that of the tube T). When this process is complete the tube X is removed and a gel column with a uniformly polymerized bottom region is obtained (see sections c, p. 21 and b, pp. 69 ff.). The tube X has a further function, namely to act as a support for the plexiglass device F, providing axial fixation of the cooling tube C.

In the top region of the gel column, the same two functions are fulfilled by the plexiglass plug P. This has a diameter very close to the inner diameter of the tube T and is adjustable along the tube C, thus providing a gel column of a desired length. The electrode vessel V1 consists of a polyethylene dish about 15 cm in diameter and about 8 cm in depth. A hole is made in its bottom snugly fitting around the tube T. Stable fixation is obtained by a collar L welded on to the bottom of the dish and an O-ring, R3, ensures an efficient sealing. The vessel is supported by a metal ring (not shown in Fig. 1) attached to a stand. The other electrode vessel V2 (made of glass and not visible in Fig. 1) has its upper rim immediately underneath this ring, permitting the tube T to be immersed in buffer to a level above the upper surface of the gel column. A cooling device and an electric stirrer are also present in this vessel. During electrophoresis ice-water from a reser-

voir is continuously pumped through this cooling system. The same reservoir also supplies water to the cooling tube C.

A third circulation pump is used for mixing the anolyte and catholyte; the intention being to minimize the occurrence of local changes in the buffer composition. The inlet for buffer pumped from the vessel V2 is at I and the outlet for the flow back to this vessel is at O.

b. *Preparation of the Monomer Solution*

The chemicals used were: (I), acrylamide (no. 5521, Eastman Organic Chemical, Rochester 3, N.Y., USA); (II), N,N′-methylenebisacrylamide (Eastman, no. 8383); (III), N,N,N′,N′-tetramethylethylenediamine or TEMED (Eastman no. 8178); (IV), ammonium persulphate; (V), tris-(hydroxymethyl)aminomethane; and (VI), HCl.

Tris-HCl buffer was employed for the runs. Using this buffer as a solvent (chilled to about 4°C) two different solutions were prepared; the first contained the two acrylamides and the accelerator TEMED, the second the initiator ammonium persulphate. Immediately before the preparation of the gel column the two solutions were thoroughly deaerated and carefully mixed in equal volumes in a flask flushed with nitrogen.

c. *Preparation of the Gel Column*

The tubes T and X were held in position by a dialysis membrane wetted and wrapped several times around the tubes and fixed with rubber bands. The lower end of the tube X was closed by a rubber stopper S. The joint tubes were immersed in a cylinder with ice-water and fixed in a vertical position. The level of the ice-water was above the intended position for the upper surface of the column to be prepared. To prevent the passage of water into the tubes through the dialysis membrane, this was prior to the immersion covered with a water-resistant tape.

The tube C was inserted, and the outer ice-water was continuously pumped through this cooling device.

The joint tubes X and T were flushed with nitrogen and carefully filled up to the brim with the monomer solution. To avoid aeration during this process nitrogen was continuously bubbled through the solution, the inlet for the gas bing kept close to the stopper S. Finally the plug P was brought in position.

After the polymerization process was terminated the column was fixed upside down and the tape and the dialysis membrane were removed. A thin wire of copper was used to cut the gel between the tubes X and T. The removal of the plug P completed the packing procedure. (Before the electrophoresis the plug was moved to the upper part of the tube C, above the brim of the tube T.)

d. *Assembly of the Electrophoresis Apparatus*

Depending on the construction principle the continued assembly of the apparatus (after the packing procedure) had to be performed in the following way. Attached to the tube T were the electrode vessel V1, part E1 of the elution chamber, the nylon net N and the ring K. Thereafter the rest of the elution chamber was screwed on to part E1 and the whole chamber was inserted into a piece of dialysis tube (length about 8 cm). By means of a rubber band the tube was closed in its lower end and held in place by an outer groove M in part E2. The bag obtained in this way was packed with polyacrylamide or agarose gel granules.[1]

To get a uniform hydrodynamic and electric flow through the elution chamber it is important that all its cavities are filled with granules. To avoid formation of air bubbles in the chamber this was immersed in buffer during the packing procedure. The packing was terminated by closing the bag with a rubber band knitted around the tube T just above the elution chamber.

The next step was to connect the tubes H with narrow polyethylene tubes for the transportation of the eluate via a peristaltic pump to a fraction collector. To minimize the inevitable deterioration in the electrophoretic resolution inherent in all elution technique, the inner diameter of the tubes was only 0.03 cm and the lengths of the tubes were kept at a minimum.

Finally the column tube T was immersed in the electrode vessel V2. A rectifier with two platinum electrodes rendered the equipment complete.

B. Pituitary LH

Fractionation by DEAE-cellulose chromatography of human pituitary concentrates prepared according to section a : 1 (pp. 15 f.) provided a major unadsorbed fraction rich in LH activity (see section a, pp. 34 f.). This fraction was subjected to further purification by molecular sieve chromatography on Sephadex G-100 in 0.2 *M* sodium acetate buffer, pH 4.35, also containing 0.5 *M* sodium chloride. Prior to chromatography the fraction was concentrated by ultrafiltration [11] and dialyzed extensively against the sodium acetate buffer. The precipitate which formed was collected by centrifugation and discarded.

After two consecutive runs on Sephadex G-100 columns the fraction enriched in LH activity was chromatographed on SE-Sephadex® C-50

[1] Granules of the former gel type were prepared in the buffer to be used for the electrophoresis and from the chemicals mentioned above (p. 21). The amounts per 100 ml of solution were: (I) 20 g; (II) 0.5 g; (III) 0.07 g and (IV) 0.1 ml. The granules were obtained by pressing the gel through a 60-mesh net [18]. The agarose granules were kindly prepared in a spherical shape by Dr S. Hjertén [19]. The concentration of the agarose was 4 %.

(Pharmacia Fine Chemicals, Uppsala, Sweden). The gel was equilibrated with 0.01 *M* potassium phosphate buffer, pH 7.0, and elution was performed at this pH-value by stepwise increases in the buffer concentration.

III:2. *Hormone Assay*

A. FSH

Biological assays of FSH activity were performed by the HCG-augmentation method in immature female rats according to Steelman and Pohley [20]. The total dose of HCG (human chorionic gonadotropin) administered to each rat together with the sample was 40 I.U. (international units). For immunological assays the radioimmunosorbent technique of Wide and Porath [103], as modified by Wide *et al.* [21] was utilized.

B. LH

Biological LH activity was determined by the seminal vesicle test in immature intact male rats as described by van Hell *et al.* [22]. Radioimmunological assays were performed according to the technique described by Wide and Porath [103].

C. GH

Biological assays of GH were made according to Greenspan *et al.* [23], based on the increment of the width of uncalcified tibial cartilage in hypophysectomized rats. Radioimmunological assays were performed according to the technique of Westphal *et al.* [24].

D. Doses

All doses of unknowns were based on absorbance measurements at 280 mμ, assuming $A_{1\,cm}^{1\%} = 10$ (see section III:6, p. 31). Doses of preparations used as standards (see below, section E) were expressed either by weight or in activity units.

All dilutions were performed with 0.01 *M* potassium phosphate buffer, pH 7.0, containing 0.9 per cent sodium chloride and also 0.015 per cent bovine albumin. The samples were then sent to the Academic Hospital in Uppsala where all the different assay procedures were carried out. The bioassays were performed by Professor Carl Gemzell, the radioimmunological FSH and LH assays by Dr Leif Wide and the radioimmunological GH assay by Dr Otto Westphal.

E. Standards

Assay data have to be expressed in terms of some standard preparation, assayed at the same time and under the same conditions as the unknowns. If the aim is only to compare the reproducibility of some purification procedure a local standard will be sufficient. To be able, however, to compare the results with those obtained in other laboratories, it is necessary to use a standard, which is commonly available. The biological FSH potencies in this study have been expressed in terms of the ovine pituitary preparation NIH-FSH-S2 (kindly supplied by The Endocrinology Study Section, National Institutes of Health, Maryland, USA). Recently and International Reference Preparation (2nd IRP) prepared from human postmenopausal urine was established [25] (available from the Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, England). One ampoule of this standard contains by definition 40 i.u. of FSH and 40 i.u. of LH activity (i.u. – international unit). Conversion factors to express the potencies of the NIH ovine preparations in i.u. of FSH per mg have been reported [26, 27] and have also been determined in the present study (see section IV:2, pp. 48 f.). For radioimmunological assays of FSH, the 2nd IRP was utilized as a standard (ovine standards cannot be used in this technique).

LH was biologically assayed against HCG, the activities being expressed as I.U. of HCG per mg. Later on a factor was determined for the conversion of these values into i.u. of LH activity per mg (se section IV:2, pp. 48 f.). The 2nd IRP was used as reference in the radioimmunological determination of LH activity. As GH was intended primarily for clinical purposes potencies were measured against a local standard.

F. Design of the Bio-Assays for FSH and LH

Each assay included two doses of the reference preparation as well as of the unknowns. In the FSH assay six rats were assigned to each dose, the log dose ratio being invariably 0.301. In the LH assay five rats were used in each dosage group and the log dose ratio was 0.477.

Calculation of potencies, fiducial limits of error and validity tests were performed as described by Gaddum [28] and by Finney [29], by means of an electronic computer. Replicate potency determinations were combined and the potency given by the geometric mean [30]. Potencies of fractions prepared by the same procedures but at different occasions were also combined and expressed by their arithmetic mean.

III:3. *Chemical Characterization of FSH*

A. Amino Acid Analysis

Hydrolyzed samples of pituitary and urinary FSH or of their oxidized or alkylated derivatives were analyzed by the method of Spackman *et al.* [31] with a Spinco Model 132 amino acid analyzer.

Hydrolysis in half concentrated HCl at 110° was performed as described by von Hofsten *et al.* [32]. Total cystine plus cysteine was converted to cysteic acid by the performic acid oxidation procedure of Moore [33]. The presence of free sulfhydryl groups was investigated by alkylation with iodoacetic acid in the Tris-EDTA-8M urea medium described by Crestfield *et al.* [34].

The analysis data were used to calculate the most probable molecular weight of the protein moiety of pituitary and urinary FSH according to the following general method. The procedure is based upon two assumptions; first that the samples to be hydrolyzed consist of only one molecular species, and second that all amino acids can be considered as determined by the same accuracy. (An average error of $\pm 3\,\%$ can be calculated from the data given by Spackman *et al.* [31], when analyzing mixtures of amino acids in amounts corresponding to those used (0.25–0.06 micromoles of each acid) in the present study. An error in the range of 3 % is therefore assumed in the determination of each amino acid, also when dealing with protein hydrolysates.)

$$\text{Let} \quad (A_1)_{y_1}(A_2)_{y_2} \ldots (A_n)_{y_n} \rightarrow y_1 A_1 + y_2 A_2 \ldots y_n A_n$$

represent the reaction for the hydrolysis of a protein molecule (or protein moiety of a conjugated molecule) to amino acids. $A_1, A_2 \ldots A_n$ stand for different free amino acids as well as for the corresponding amino acid residues; y_1, $y_2 \ldots y_n$ denote the integers by which the amino acid residues enter into the protein molecule. The experimentally determined amounts of the respective amino acids (in micromoles) are represented by $\mu_1, \mu_2 \ldots \mu_n$. Wellknown stoichiometry gives

$$\frac{\mu_1}{y_1} = \frac{\mu_2}{y_2} = = \frac{\mu_n}{y_n} = \frac{1}{k}, \tag{1}$$

or

$$y_i = \mu_i k \quad (i = 1, 2 \ldots n), \tag{2}$$

where k is a constant and $1/k$ is the number of micromoles of protein being analyzed. In the ideal case (a pure protein and no error in the determination of μ_i) it exists consequently such a k-value (and its multiples) that all y-values are provided as integers. This means that in a graphical representa-

tion of the n equations (2) (with k as abscissa and y as ordinate), there exists a line, parallel with the y-axis and giving n intersections, all with an integral number as y-coordinate.

Owing to the error in the determination of μ there is in practice no such line. The problem is therefore to determine a line, parallel with the y-axis and giving such y-coordinates that as a total the relative deviation from nearest integral numbers for the different amino acids is minimized.

In the study of this problem only those amino acids are considered which are stabile during hydrolysis or those whose decomposition can be accurately corrected for. As a consequence of the average inaccuracy of 3 % in the μ-values no amino acids with y-values greater than 16 are considered. Otherwise the error may exceed 0.5 amino acid residue and it is then no longer justified to consider the nearest integer as the most probable one.

For each amino amino acid which fulfills the above criteria, those k-values are calculated from eqn. (2), which correspond to $y = 1, 2 \ldots 16$. (A crude estimation of the molecular weight by some independent method is very helpful to limit the range of k-values which need to be investigated.) The k-values were plotted in principle as shown in Fig. 12, which illustrates an actual case. The amino acids are ranked after their μ-values (and accordingly also after their y-values), with the lowest value nearest to the k-axis. If, as in Fig. 12, there is more than one value for k around which the spread of relative values for all amino acids is small, they all have to be considered. The best line through each of these set of points is that with a k-value being the arithmetical mean ($\bar{k}$) of the k-values for the points involved. (This can be shown by the method of least squares.) To be able to descriminate between these different lines the coefficient of variation ($s/\bar{k}$) is calculated in each case. The most probable $\bar{k}$-value ($\bar{k}'$) is that which minimizes this coefficient. In the expression above s is the standard deviation and accordingly

$$s = \frac{1}{\sqrt{m-1}} \sqrt{\sum_{i=1}^{m} (k_{A_i} - \bar{k})^2}, \tag{3}$$

where k_{A_i} is the k-value relevant for the amino acid A_i and m is the number of different amino acids involved in the calculation. The number of amino acid residues (Y_i) is then calculated from the equation

$$Y_i = \mu_i \bar{k}' \tag{4}$$

for all acids (n) determined by the analysis. The molecular weight then equals $\sum_{i=1}^{n} y_i R_i$ where y_i is the nearest integer to Y_i and R_i is the residual molecular weight of the amino acid A_i.

In general more than one analysis of a substance is performed and $\mu_1, \mu_2 \ldots \mu_n$ then represent averaged values. The prerequisite for such a direct comparison between data from different runs is, that an equal amount of sample is analyzed in each experiment. This is often not the case and the calculation of averaged values therefore has to be preceded by the application of correction factors in order to normalize all values to the same amount of sample. In the present study this normalization was performed by selecting one of the runs (with a 72-h hydrolysate) as a reference. The values for each amino acid determined with confidence from the other analyses, were then divided by the value for the same amino aicid, as determined from the reference run. In general most amino acids determined by the analysis procedure were considered in this calculation. Excluded were those which are known or suspected to decompose (threonine, serine, cystine, methionine, tyrosine) or to have a slow liberation during acid hydrolysis (valine, isoleucine). Furthermore, the treatment of the sample prior to analysis can determine, whether the value for a particular amino acid should be considered as reliable or not. One example is lysine which is adequately determined from a normal 24-h hydrolysate but not from an alkylated 24-h hydrolysate. It should finally be stressed that in this calculation there are no reasons to exclude those amino acids which occur in the molecule in numbers greater than 16.

By the normalization procedure all amino acids considered from a given run were allotted a correction factor. These factors were averaged and thus provided the factor used to correet the values for *all* amino acids determined in that particular analysis. The corrected values for those amino acids, which fulfill the above criteria (page 26), are then utilized in calculating the $\bar{k}'$-value. In eqn. (4) μ_i now stands for the averaged corrected value of the amino acid A_i. If A_i is decomposed during hydrolysis, μ_i is the value obtained by first-order reaction extrapolation to zero hydrolysis time of the averaged corrected values from 24-h hydrolysates and from 72-h hydrolysates. Finally, if A_i is slowly liberated, μ_i is the average of corrected values from 72-h hydrolysates.

B. Molar Extinction Coefficient

The molar extinction coefficients (ε) of pituitary and urinary FSH at 280 mμ and at neutrality were calculated in the following way from parameters determined in connection with the amino acid analyses (see section A, pp. 25 ff.).

Protein samples intended for analysis were dialyzed against 0.001 *M* potassium phosphate buffer, pH 7.0, or against distilled water. Following

the dialysis the absorbance $A_{1\,cm}^{280\ m\mu}$ was measured and a known volume V (μl) of the sample was hydrolyzed. The hydrolysate was evaporated to dryness and then dissolved in 5.0 ml citrate buffer, pH 2.2 [31]. Of this solution 2.0 ml were applied on each of the two columns used in the procedure of Spackman *et al.* [31]. It follows that according to Beer's law the amount of protein hydrolyzed (μ micromoles) can then be written as

$$\mu = \frac{A_{1\,cm}^{280\ m\mu} V}{\varepsilon}. \tag{5}$$

The dimension of ε is μmol^{-1} μl cm^{-1}, equivalent to the commonly used mol^{-1} 1 cm^{-1}. According to the above considerations the amount of hydrolysate used for a complete analysis corresponds to 0.4 μ micromole of protein. It then follows from eqn. (1) that

$$0.4\,\mu = \frac{1}{k}. \tag{6}$$

Eqns. (5) and (6) give

$$\varepsilon = 0.4\ A_{1\,cm}^{280\ m\mu}\, V k. \tag{7}$$

If ε is calculated from a number (n) of different runs, normalized as described in connection with the amino acid analyses (p. 27), eqn. (7) can be written

$$\varepsilon = \frac{0.4\,\bar{k}'}{n} \sum_{i=1}^{n} \left(\frac{A_{1\,cm}^{280\ m\mu} V}{f} \right)_i. \tag{8}$$

In eqn. (8) $\bar{k}'$ has the significance earlier discussed and f are the different normalization factors. (By definition the factor equals 1 for the reference run.)

C. Estimation of Tryptophan

Tryptophan is completely destroyed with acid hydrolysis of FSH and consequently not determined with the procedure of Spackman *et al.* [31]. The number of tryptophan residues (y_{Try}) in FSH was estimated from the difference between the molar extinction coefficient of the hormone (ε_{FSH}) and the contributions in absorbance given by the tyrosine and cystine residues according to eqn. (9) (see ref. 35).

$$y_{Try}\,\varepsilon_{Try} = \varepsilon_{FSH} - (y_{Tyr}\,\varepsilon_{Tyr} + y_{Cys}\,\varepsilon_{Cys}). \tag{9}$$

The value for ε_{FSH} (pituitary or urinary FSH) refers to 280 mμ at about pH 7 and was calculated in connection with the amino acid analyses (section B, pp. 27 f.). ε_{Try} (5500 mol^{-1} 1 cm^{-1}), ε_{Tyr} (1100 mol^{-1} 1 cm^{-1}) and ε_{Cys}

(125 mol^{-1} 1 cm^{-1}) are the molar extinction coefficients of the respective amino acids at 280 mμ and pH 6. The values for tyrosine and tryptophan are those given by S. Malik (unpublished), as cited by Wetlaufer [35] and also by Schellman and Schellman [36], and the value for cystine is calculated from data given by Beaven and Holiday [37]. Finally, y_{Tyr} and y_{Cys} are the number of residues of these amino acids present in FSH according to the amino acid analyses.

D. Carbohydrate Analysis

D:1. *Hexose*

Analyses were performed by the orcinol-sulfuric acid procedure as described by Svennerholm [38]. The amount of hexose was determined from a standard curve employing mannose (Pfanstiehl) as a standard.

D:2. *Hexosamine*

The determination of hexosamine was performed according to Spackman *et al.* [31] in connection with the amino acid analysis.

D:3. *Sialic Acid*

The thiobarbituric acid procedure of Warren [39] was employed for the determination of sialic acid. The results were expressed in terms of N-acetylneuraminic acid (Burr) used as a standard.

E. Partial Specific Volume

The partial specific volume ($\bar{v}$, g^{-1} cm^3) of FSH was calculated by eqn. (10) from the contributions of the amino acid and carbohydrate residues [40, 41] found in the hormone molecule

$$\bar{v} = \frac{\sum_{i=1}^{n} y_i R_i \bar{v}_i}{\sum_{i=1}^{n} y_i R_i}, \qquad (10)$$

where y_i = the number of residues of the amino acid A_i as determined directly or indirectly (tryptophan) by the amino acid analyses (Tables VIII, p. 51 and IX, p. 52) and also the number of residues of the different carbohydrate components (Table X, p. 53).

R_i = the residual weight of A_i and of the carbohydrate components.

$\bar{v}_i$ = the partial specific volume of the residue A_i and of the carbohydrate residues. The values (at 25°C) given by Cohn and Edsall [40] and by Gibbons [41] were employed.

n = the number of different amino acids and carbohydrates included in the analyses.

It should be emphasized that $\bar{v}$ is independent of the molecular weight but depends on the relative amounts of the different constituents (this also follows from eqn. (2) $y_i = \mu_i\, k$ in section A, pp. 25 ff.).

III:4. *Physical Characterization of FSH*

A. Sedimentation Constant

Studies of the sedimentation velocity were carried out in a Spinco model E ultracentrifuge, equipped with Schlieren optics, including a phase plate as the Schlieren diaphragm. For each of the two hormone preparations the sedimentation coefficient was calculated at three different protein concentrations. The sedimentation constant ($s^0_{20,w}$) was obtained by correction of these values to the viscosity and density of water at 20°C, followed by a linear extrapolation to zero protein concentration [42, 43].

B. Molecular Weight

The molecular weights of pituitary and urinary FSH were calculated from sedimentation equilibrium runs in the ultracentrfiuge. In these experiments the centrifuge was equipped with Rayleigh interference optics [44] and initial protein concentrations (in fringes) were determined from runs utilizing a double sector synthetic boundary cell. The equilibrium runs were performed in a double sector cell. The apparent molecular weight was calculated from the Svedberg [42] formula

$$M = \frac{2\,RT}{(1-\bar{v}\varrho)\omega^2\, 10 \log c} \quad \frac{d \log c}{d r^2}, \tag{11}$$

where the symbols have their usual significance [42, 43]. Analysis of data by plotting the logarithm of concentration (c) in terms of Rayleigh fringes as a function of the square radial distance (r^2) provided the factor $(d \log c)/(dr^2)$. The partial specific volume ($\bar{v}$) was calculated according to section E, pp. 29 f.

C. Free Zone Electrophoresis

The two hormone preparations were investigated for electrophoretic homogeneity in a Hjertén apparatus for free zone electrophoresis [45].

D. Ultraviolet Absorption Spectrum

The ultraviolet spectra of pituitary and urinary FSH were examined both in neutral and alkaline solutions.

III:5. *Immunological Studies of FSH*

A. Antisera

The antisera were raised in guinea pigs by three consecutive intramuscular injections. The antigen was mixed with Freund's adjuvant and three portions of about 0.5 mg of antigen was injected into each animal at weekly intervals. The anti-serum used in this study was raised against a pituitary FSH preparation with a biological activity of 6000 i.u. per mg. Injections of urinary FSH preparations of different purities did not result in any detectable formation of anti-urinary FSH.

B. Gel Diffusion

Ouchterlony gel diffusion analysis [46] was carried out in 0.3 *M* potassium phosphate buffer, pH 8.0 [47]. The diffusion was performed in 1.35 % agarose gel, utilizing agarose prepared according to Hjertén [48]. The advantage of using agarose gels instead of agar gels in this technique has been pointed out by Brishammar *et al.* [49].

III:6. *Protein Determination*

Calculations of yields from the different separation steps and of doses for the assays were based on spectrophotometric readings at 280 mμ. The calculations were performed under the assumptions that Beer's law obtains for the various solutions giving absorbance values $A^{1\,\%}_{1\,\mathrm{cm}} = 10$ (1 % solution, 1 cm light path).

Lyophilization or precipitation of proteins with organic solvents were generally avoided throughout the fractionation procedure and protein solutions were concentrated by ultrafiltration. This was made either by the arrangement described by von Hofsten and Falkbring [11] or by utilization of collodion sacs, available from Membranen-Gesellschaft, Göttingen, Germany. The collodion sacs were used in cases where the desired volume was 3 ml or less.

IV. EXPERIMENTS AND RESULTS

IV:1. *Purification*

A. Pituitary and Urinary FSH

A:1. Extraction and Ammonium Sulphate Fractionation

a. *Pituitary Material*

a:1. *Whole frozen glands*

An average weight of one human pituitary gland is 0.62 g, as estimated from many batches. In general 300–400 pituitaries were processed each time. This meant that the volumes of extracts A and B were of the order of 1.5 and 3.5 liters, respectively.

The preparation procedure was carried out without any unnecessary interruptions and the time for its completion was ultimately set by the capacity of the available centrifuge. The capacity of the centrifuge used in this laboratory was 1.3 liters and 12–15 h were required for obtaining a hormone concentrate ready for dialysis.

The pH value was kept between 6 and 7 throughout the preparation. The pH value of extract A was 6.2. Homogenization of the pituitaries thus gave rise to an increase of the original pH value of 5.7 (section a:1, pp. 15 f.). Immediately before the addition of saturated $(NH_4)_2SO_4$ solution to extract A, the pH was increased to 7.0. This was necessary in order to keep the pH value in the desired range in spite of the large amounts of $(NH_4)_2SO_4$ to be added. Actual measurements after the complete addition of this salt (saturated solution and solid) gave pH values of about 6.2. The gonadotropin-containing precipitate was dissolved in about 500 ml of distilled water and dialyzed against the 0.02 *M* phosphate buffer (p. 16). A small precipitate which formed was removed by centrifugation and discarded.

An average yield (spectrophotometrically determined) from 31 preparations was 2.80 g per 100 g of pituitary tissue, with a standard error of ± 0.41 g. Table I records the results of the biological assays of 5 preparations (7 independent assays). Expressed in terms of the 2nd IRP the mean potency is about 30 i.u. per mg. It follows that on an average about 500 i.u. of FSH activity are extracted from one gland. Biological LH activities of two preparations are listed in Table II (p. 36); each preparation being assayed on two separate occasions. The mean potency is 63 i.u. per mg corresponding to about 1100 i.u. of LH activity from one gland.

Table I. Purification of human pituitary FSH.
Assay of various fractions for FSH activity.

Method of preparation	Fraction	Relative potency: Biological assay Units/mg[a]	Mean units/mg	Mean i.u./mg	Radioimmunological assay[b] i.u./mg	Mean i.u./mg	Index of discrimination [51]
Extraction and ammonium sulphate fractionation (section a:1, pp. 32 ff.)	P 58	1.4 (1.1–1.8)			34		0.94
	P 58	1.5 (1.1–1.9)					
	P 69	1.4 (0.9–1.9)					
	P 79	1.4 (1.1–1.7)	1.3	*29*			
	P 84	1.3 (1.0–1.6)					
	P 85	1.2 (0.9–1.5)					
	P 85	1.2 (1.0–1.5)					
DEAE-cellulose chromatography (section a, pp. 34 f.)	D 116	12 (10–14)					
	D 119	12 (10–16)					
	D 119	14 (9–20)					
	D 128	13 (11–15)	13	*300*	260		1.2
	D 128	15 (12–22)					
	D 131	15 (12–20)					
	D 131	13 (10–17)					
Molecular sieve chromatography (section a, pp. 37 ff.)	*1st run*						
	F 135	140 (110–190)					
	F 135	140 (110–200)					
	F 136	140 (110–180)	150	*3300*			
	F 137	160 (140–190)					
	F 137	160 (130–210)					
	2 nd-run						
	F 124	250 (210–310)					
	F 130	290 (230–420)					
	F 138	240 (180–370)	260	*5700*			
	F 142	210 (180–240)					
	F 151	320 (230–540)					
	F 159	250 (190–330)					
Hydroxylapatite chromatography (section a, pp. 41 f.)	Ca 32	500 (320–680)					
	Ca 32	580 (470–720)					
	Ca 32	480 (340–630)	550	*12,000*			
	Ca 38	620 (510–760)					
	Ca 46	550 (430–720)					
Polyacrylamide gel electrophoresis (section b, pp. 45 f.)	E 43	690 (570–820)			13,800		
	E 43	650 (450–860)					
	E 46	510 (390–630)	630	*14,000*	13,800	14,000	1.0
	E 53	610 (420–810)			14,300		
	E 53	710 (560–860)					

[a] 1 unit – the activity in 1 mg of NIH-FSH-S2 – 22 i.u. (Table VII, p. 49). Figures in parentheses are the 95 % confidence limits.

[b] Values from ref. 21.

Preparations for clinical use were dialyzed against the volatile buffer NH_4HCO_3 and then lyophilized. Yields and specific biological activities of lyophilized preparations (calculated on a weight basis) were in general in good agreement with those of non-lyophilized preparations (calculated on readings at 280 mμ). This suggests (1) that the assumption of $A^{1\,\%}_{1\,cm} = 10$ at 280 mμ must be fairly correct for material of this purity and (2) that lyophilization *per se* did not cause any losses in biological potency.

The first point was experimentally verified by reading the absorbance at 280 mμ of solutions containing 1 mg of lyophilized material per ml. On average five preparations gave $A^{280\,m\mu}_{1\,cm} = 1.1$.

The second point was proved in the following way. The same volume of dialysate was added to each of two test tubes. The samples were chosen to contain an amount of material suitable for performing a reliable assay. One of the test tubes was put into a lyophilizer and the dried material (still in the same test tube) was again dissolved and all of it used for the assay. The contents of the other test tube constituted the control. No significant difference in the biological potencies of the two samples could be established.

a:2. *Acetone-preserved glands*

In general, the details given above apply also to acetone-preserved glands. It should be added, however, that the precipitate formed at half-saturation with ammonium sulphate is consistently very small and difficult to collect by centrifugation. Furthermore, the yeild of FSH varied considerably between batches. The highest yield of FSH obtained from acetone-treated glands was 200 i.u. per pituitary.

A:2. DEAE-Cellulose Chromatography

a. *Pituitary Material*

A preparation of an average size provided after dialysis about 6 g of the gonadotropin concentrate. The dimensions of the column used for its fractionation were 4 × 100 cm (200 ml adsorbent per g of protein). In the first step (0.02 *M* potassium phosphate buffer, pH 7.0) the column was operated at a flow rate of 15 ml/cm²/h, and fractions of about 25 ml were collected. Under these conditions about 45 % of the applied material was eluted. Fig. 2. shows a representative elution pattern; the material corresponding to the cross-hatched area had an LH activity of about 130 i.u. per mg (Table VI, p. 47) and negligible FSH activity at a dose lovel of 2 mg. It constituted about 85 % of the material recovered in this step and was further fractionated to give a highly active LH preparation (see section B, pp. 46 ff.). Biological assays of the remaining fractions from this step indi-

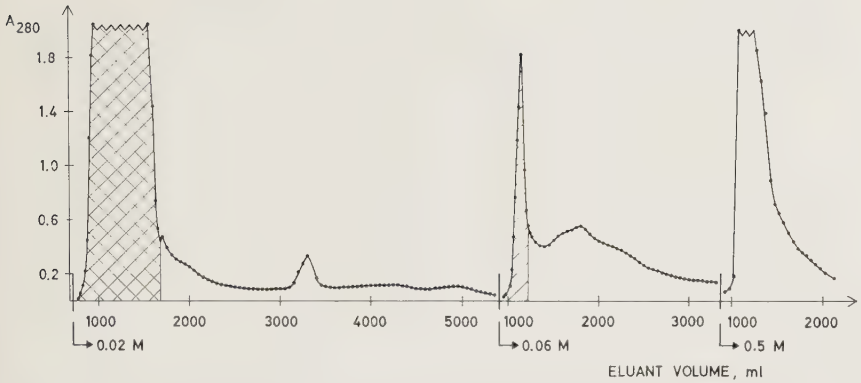

Fig. 2. DEAE-cellulose chromatography of human pituitary FSH concentrate (5.8 g). The fractions within the cross-hatched area contained the principal amount of LH activity. In this figure, as well as in Figs. 3–11, fractions within hatched areas contained FSH activity and were utilized in the progressive purification of the hormone.

cated LH activities similar to those in the main fraction and also indicated the presence of small amounts of FSH.

The main part of the FSH activity was eluted by a one-step increase in the buffer concentration to 0.06 *M*. The flow rate was altered to be 5 ml/cm²/h and fractions of about 11 ml were collected. After assaying by the Steelman-Pohley method [20], those fractions intended for further purification were pooled. In Fig. 2 these fractions are indicated by the hatched area. The pool was assayed and the yield calculated from the volume and the absorbance at 280 mμ. On average about 100 mg per 100 g of pituitary tissue were obtained. The FSH potency of the pooled fractions was about 300 i.u. per mg and the LH potency was estimated to be 32 i.u. per mg. Recoveries and assay data from a number of preparations are given in Tables I (p. 33), II (p. 36) and IV (p. 42). At a final buffer concentration of 0.5 *M* further material emerged from the column. Bio-assays did not reveal the presence in this material of any significant amounts of gonadotropic activity.

b. *Urinary Material*

The postmenopausal urine concentrate had a biological FSH potency of 8.6 i.u. per mg (mean of 3 determinations, Table III, p. 38) and a biological LH potency of 210 i.u. per mg (mean of 2 determinations, see Table II). Three equal portions of the concentrate (3 × 3.2 g) were separately chromatographed on DEAE-cellulose. To each of the portions were added 100 ml of the 0.02 *M* phosphate buffer, the material being suspended by manual stirring and insoluble components being collected by centrifugation. The precipitate was resuspended in 100 ml of the buffer and the supernatant

Table II. Purification of human pituitary and urinary FSH.

Assay of various fractions for LH activity.

Method of preparation	Fraction	Relative potency: Biological assay: I.U. of HCG/mg[b]	Mean I.U. of HCG/mg	Mean i.u./mg	Radioimmunological assay[a]: i.u./mg	Mean i.u./mg	Index of discrimination [51]
Pituitary material							
Extraction and ammonium sulphate fractionation (section a:1, pp. 32 ff.)	P 89	11 (7.6–15)	10	*63*	97	100	0.61
	P 89	9.8 (7.0–13)					
	P 90	9.1 (6.0–12)			111		
	P 90	11 (8.4–14)					
DEAE-cellulose chromatography (section a, pp. 34 f.)	D 136	3.9 (2.8–5.1)	5.2	*32*	17	18	1.8
	D 136	5.3 (4.1–6.5)					
	D 137	5.7 (3.8–7.8)			19		
	D 137	6.0 (4.2–8.8)					
Polyacrylamide gel electrophoresis (section b, pp. 45 f.)	E 43				9.7		
	E 46				25	18	
	E 53				19		
Urinary material							
Starting material (section II:2, p. 14)	H 70	34 (19–50)	34	*210*	44		4.8
	H 70	33 (25–47)					
Polyacrylamide gel electrophoresis (section c, p. 46)	E 45				5.1		
	E 47				22	15	
	E 48				18		

[a] Values from ref. 21.

[b] 1 I.U. of HCG = 6.25 i.u. (Table VII, p. 49). Figures in parentheses are the 95 % confidence limits.

decanted after centrifugation. The washing procedure was repeated once more and the three supernatants were pooled. The conductivity of the pooled solutions was higher than that of the 0.02 *M* buffer, probably due to the presence in the urinary concentrate of some inorganic salt. This discrepancy was corrected for by the addition of distilled water; finally the pH value was checked. The ultimate volume of the starting zone was approximately 400 ml and on a UV-basis 3.0 g of material were applied to the column. The column dimensions were 3.7×95 cm and fractions of about 25 ml were collected using a flow rate of 15 ml/cm^2/h. The unadsorbed material (see Fig. 3) accounted for about 30 % of the amount applied. About 60 % of the unad-

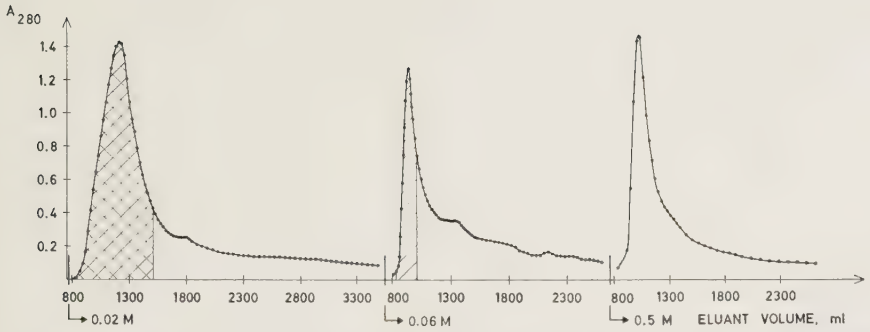

Fig. 3. DEAE-cellulose chromatography of postmenopausal urinary concentrate (3.0 g), The cross-hatched area represents the principal LH-containing fraction.

sorbed material emerged in a well-defined peak in the chromatogram (the cross-hatched area in Fig. 3), the other 40 % in two less pronounced peaks. Assays for FSH activity on material corresponding to the main peak gave no significant response for doses up to 2 mg. Material corresponding to the second and third peaks had FSH potencies of approximately 5 and 9 i.u. per mg, respectively. The LH potency of the main fraction was estimated to be 250 i.u. per mg.

Increase of the buffer concentration to 0.06 *M* resulted in the elution of the essential part of the FSH activity. The flow rate and the fraction volumes were 3 ml/cm²/h and 8 ml, respectively. The fractions within the hatched area (Fig. 3) are those of high relative potency and were selected for further purification. About 43 mg of this material were recovered per g of urinary concentrate, associated with an FSH potency of about 120 i.u. per mg. Yields and assay data from the three runs are summarized in Tables III and V (p. 44). Increasing the buffer concentration to 0.5 *M* did not provide further gonadotropin-containing material.

A:3. Molecular Sieve Chromatography

a. *Pituitary Material*

After ultrafiltration of the active fractions from the preceding step there remained from each preparation about 200 mg of protein, fractionated by molecular sieve chromatography. The volume of the starting zone was about 6 ml, the column dimensions were 220 × 2.2 cm, and fractions of 2.5–3.0 ml were collected (the buffer used and the flow rate are given in section A:3, pp. 17 f.). Figure 4 shows the protein distribution after a run performed under these conditions. The fractions selected for further purification are indicated within the hatched area. When correlated to 100 g of pituitary tissue the

Table III. Purification of human urinary FSH.

Assay of various fractions for FSH activity.

Method of preparation	Fraction	Relative potency: Biological assay Units/mg[a]	Mean Units/mg	Mean i.u./mg	Radioimmunological assay[b] i.u /mg	Mean i.u./mg	Index of discrimination [51]
Starting material (section II:2, p. 14)	H 70	0.38 (0.30–0.51)					
	H 70	0.40 (0.30–0.55)	0.39	*8.6*		10	0.86
	H 70	0.39 (0.33–0.48)					
DEAE-cellulose chromatography (section b, pp. 35 ff.)	HD 12	5.1 (3.9–6.5)					
	HD 12	5.2 (4.0–8.0)					
	HD 14	5.4 (4.3–6.7)	5.4	*120*	130		0.92
	HD 15	5.7 (4.4–7.7)					
	HD 15	5.3 (3.9–8.6)					
	1st run						
Molecular sieve chromatography (section b, p. 40)	HF 18	12 (8.3–24)					
	HF 18	22 (9.6–14)					
	HF 19	12 (10–14)	12	*260*			
	HF 19	11 (9.4–14)					
	HF 19	12 (8.7–17)					
	2 nd run						
	HF 20	19 (16–23)					
	HF 20	17 (13–21)					
	HF 21	16 (13–19)	17	*380*			
	HF 21	19 (15–27)			290		1.3
	HF 21	17 (13–21)					
Hydroxylapatite chromatography (section b, p. 43)	Ca 37	32 (23–40)					
	Ca 37	27 (20–35)					
	Ca 40	32 (23–37)	28	*620*			
	Ca 42	27 (21–34)					
	Ca 42	24 (16–31)					
Polyacrylamide gel electrophoresis (section c, p. 46)	E 45	35 (31–40)					
	E 45	39 (32–49)					
	E 47	33 (28–39)	35	*780*	570	620	1.2
	E 48	33 (25–44)			660		
	E 48	36 (29–45)					

[a] See footnote *a*, p. 33.

[b] Values from ref. 21.

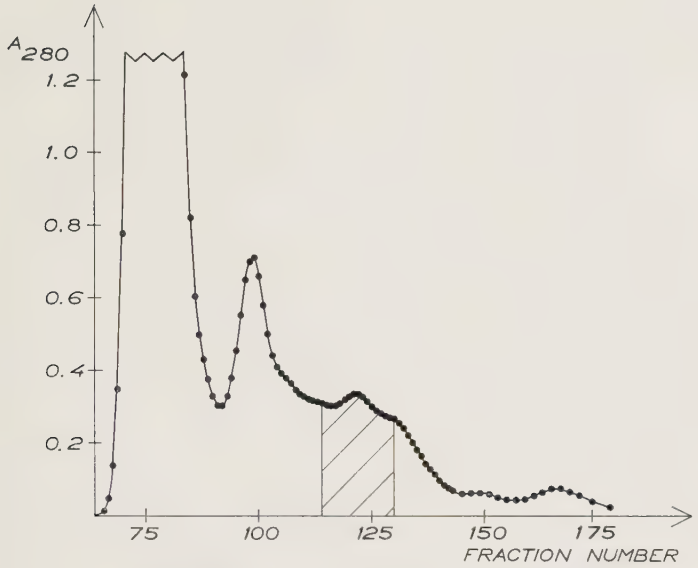

Fig. 4. Molecular sieve chromatography on Sephadex G-100 of pituitary FSH preparation (225 mg), following DEAE-cellulose chromatography (Fig. 2).

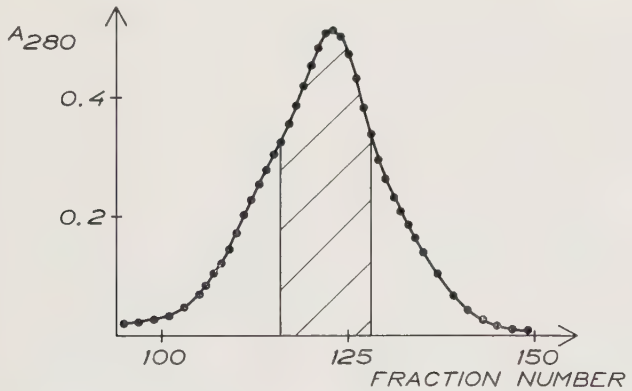

Fig. 5. Rechromatography on Sephadex G-100 of pituitary FSH preparation (26 mg) obtained as shown in Fig. 4.

recovery in this step was approximately 7 mg, associated with an FSH potency of about 3300 i.u. per mg. More precise values are given in Tables I (p. 33) and IV (p. 42). Protein recoveries were repeatedly 94–98 %.

This active material was concentrated and rechromatographed under identical conditions. In order to decrease the percentage losses pooled material from two or three runs were used in each rechromatographic experi-

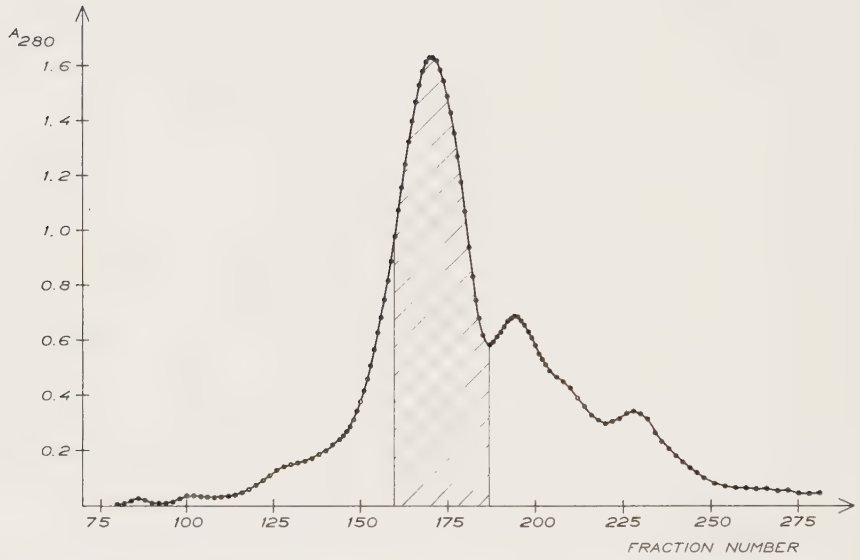

Fig. 6. Molecular sieve chromatography on Sephadex G-100 of urinary FSH preparation (171 mg) prepared by DEAE-cellulose chromatography (Fig. 3).

ment. In Fig. 5 the effluent profile is recorded and the fractions intended for further purification are also indicated. Prior to concentration the yield was estimated to be 3 mg per 100 g of pituitaries (Table IV). The potency of the active pooled fractions was about 5700 i.u. per mg (see Table I).

b. *Urinary Material*

Following bio-assays and concentration by ultrafiltration, pooled fractions (385 mg) from the three runs on DEAE-cellulose yielded 342 mg in a volume of 10 ml. This material was divided into two equal portions, each being subjected to molecular sieve chromatography under conditions identical to those discussed for the pituitary material. Figure 6 shows the effluent profile after development of the chromatogram. About 75 mg were recovered from each of these runs which corresponds to a yield of about 17 mg per g of the original urinary concentrate. The FSH potency was estimated to be 260 i.u. per mg (see Tables III and V, p. 44, for further details). The two active 75 mg portions were separately concentrated and yielded each about 70 mg. Rechromatography under identical conditions resulted in the elimination of further inactive material and of material with low relative potency (Fig. 7). The yield from each of these runs was about 40 mg which is equivalent to about 9 mg per g of the urinary concentrate. The FSH activity was about 375 i.u. per mg (Tables III and V).

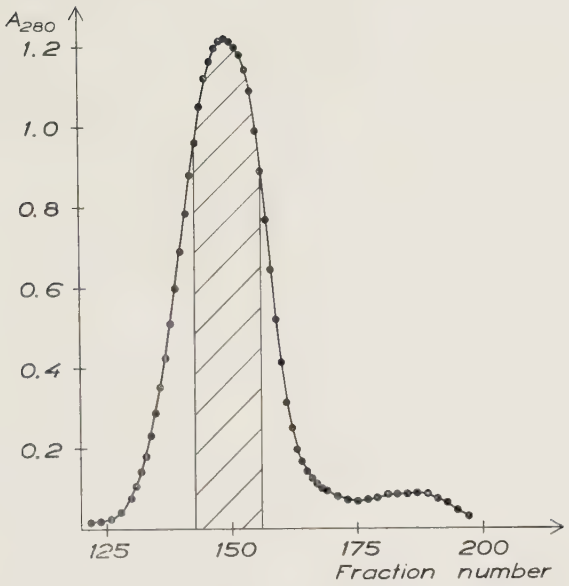

Fig. 7. Rechromatography on Sephadex G-100 of urinary FSH preparation (70 mg) obtained as shown in Fig. 6.

A:4. Hydroxylapatite Chromatography

a. *Pituitary Material*

The pooled fractions from the preceding step were concentrated to a volume of 5–6 ml and then dialysed against 0.001 *M* potassium phosphate buffer, pH 7.8. The amount of material applied in each experiment was about 12–15 mg (this represents the material recovered from 2–3 pituitary preparations).

The diameter of the column was 2.0 cm and it was packed to contain 25–30 ml of adsorbent. The flow rate was consistently low and never exceeded 3 ml/cm²/h. If the value was less than 1.5 ml/cm²/h an increase in flow rate was obtained by the use of a pressure head (0.2 atm.). Fractions of about 3 ml were collected.

The starting buffer eluted only a small and inactive portion of the material applied (Fig. 8). In order to detect the presence of minor components spectrophotometric readings were also performed at 230 mμ where the absorbance was about 5 times higher than at 280 mμ.

The FSH activity was eluted with 0.01 *M* buffer and the fractions within the hatched area (Fig. 8) were pooled. The recovery was estimated to be 0.6 mg per 100 g of pituitary tissue with an FSH activity of approximately 12,000 i.u. per mg (Tables I, p. 33, and IV, p. 42). The fractions constituting the tailing edge (Fig. 8) had significantly lower potencies. Further

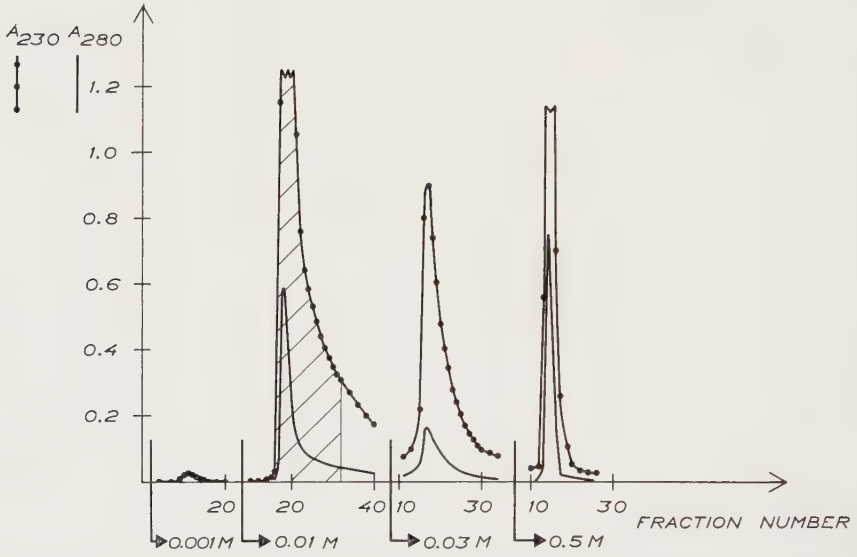

Fig. 8. Hydroxylapatite chromatography of pituitary FSH preparation (13 mg) from molecular sieve chromatography (Fig. 5).

material emerged from the column with successive use of 0.03 *M* and 0.5 *M* buffer. No biological response was obtained at dose levels which for the former material were 3 times higher and for the latter 10 times higher than the levels appropriate for assaying material eluted with the 0.01 *M* buffer.

Table IV. Purification of human pituitary FSH.

Mean biological FSH potencies, weight yields and recovered activities.
(Yields are given as means ±S.D. with the numbers of observations in parentheses.)

Method of preparation	Relative potency, i.u./mg[a]	Weight yield, mg/100 g of pituitaries	Recovered activity: i.u./ 100 g of pituitaries	Recovered activity: %
Extraction and ammonium sulphate fractionation	29	2800±400 (31)	81,000	
DEAE-cellulose chromatography	300	100±9 (12)	30,000	37
Molecular sieve chromatography:				
1st run	3300	6.5±1.1 (11)	21,000	26
2nd run	5700	2.6±0.3 (6)	15,000	18
Hydroxylapatite chromatography	12,000	0.63±0.12 (4)	7600	9.3
Polyacrylamide gel electrophoresis	14,000	0.29±0.06 (3)	4100	5.0

[a] Values from Table I, p. 33.

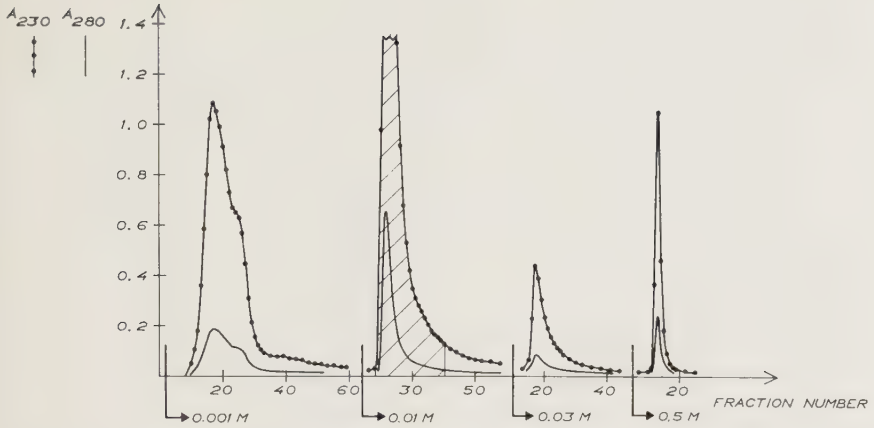

Fig. 9. Hydroxylapatite chromatography of urinary FSH preparation (23 mg), following molecular sieve chromatography (Fig. 7).

b. *Urinary Material*

The active material from the reruns on Sephadex G-100 was pooled and concentrated by ultrafiltration to a volume of about 12 ml. About 4 mg (5–6 %) of this concentrate were removed for other experiments and the rest divided into three portions of 20–25 mg which were separately chromatographed on hydroxylapatite. The diameter of the column was 2.8 cm and the experimental details were as described in the preceding section.

The essential part of the FSH activity was eluted at a buffer concentration of 0.01 *M* (Fig. 9). About 35 % of the protein applied was recovered at this buffer concentration. This corresponds to about 3 mg per g of urinary concentrate (Table V, p. 44). The FSH potency of the pooled fractions was about 620 i.u. per mg (Table III, p. 38).

Unretarded fractions (0.001 *M* buffer) did not contain any significant amounts of activity when assayed in doses 3 times higher than those used for assays of the active pool. This was also the case for material eluted with 0.5 *M* buffer and assayed in doses increased by a factor of 10.

The material eluted with 0.03 *M* buffer had some FSH activity associated with it. Both the potency and the recovery were about 20 % of those of the most active pooled fractions (0.01 *M* buffer). Accordingly the number of activity units accounted for in the 0.03 *M* step was only about 4 % of the number of units recovered by elution with the 0.01 *M* buffer.

A : 5. Polyacrylamide Gel Electrophoresis

The electrophoretic separations were performed in 0.05 *M* Tris-HCl buffer at pH 8.7 with the anode in the vessel V2. The gel was polymerized from 5

Table V. Purification of human urinary FSH.

Mean biological FSH potencies, weight yields and recovered activities.
(Yields are given as means ±S.D. with the numbers of observations in parentheses.)

Method of preparation	Relative potency, i.u./mg[a]	Weight yield, mg/g of starting material	Recovered activity	
			i.u./g of starting material	%
Starting material	8.6	1000	8600	
DEAE-cellulose chromatography	120	43±4 (3)	5200	60
Molecular sieve chromatography:				
1st run	260	16.7±0.3 (2)	4300	50
2nd run	380	8.6±0.2 (2)	3300	38
Hydroxylapatite chromatography	620	3.0±0.4 (3)	1900	22
Polyacrylamide gel electrophoresis	780	0.85±0.05 (3)	660	7.7

[a] Values from Table III, p. 38.

per cent (w/v) acrylamide, 0.13 per cent (w/v) N,N′-methylenebisacrylamide, 0.1 per cent (v/v) TEMED and 0.07 (w/v) ammonium persulphate. Using the terminology introduced by Hjertén and Mosbach [50] this means that the gel had the composition T = 5.13 %, C = 2.53 %, where T is the total concentration of the monomers and C the concentration of the cross-linking compund N,N′-methylenebisacrylamide. The height of the gel column was 25 cm. The total current was 35 mA of which about 3 mA passed the shunt containing the buffer-mixing bump. The buffer flow through the elution chamber was 9–10 ml per hour and fractions of about 1.5 ml were collected. Under these conditions both pituitary and urinary FSH emerged from the column after approximately 20 h.

Before application of the starting zone ammonium persulphate and impurities having ultraviolet absorption were electrophoretically removed. The duration of such preruns and the current used were the same as in runs with a loaded column, except that the direction of the current was reversed. The reason for these conditions were the observations (1) that the passage of current through the gel made its top and bottom surfaces slightly convex in the direction towards the anode and (2) that the original flat gel surface was apparently restored by passing the same amount of electricity in the reversed direction. This performance of the prerun prevents clogging of the small porous discs in the elution chamber and gives optimal resolution of the migrating zones.

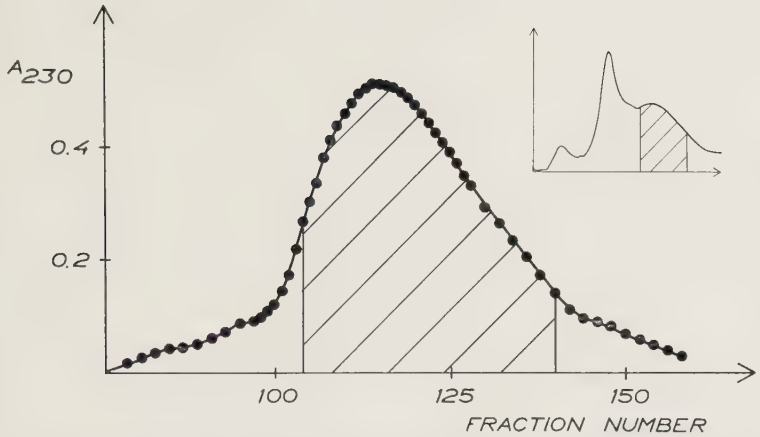

Fig. 10. Polyacrylamide gel electrophoresis of pituitary FSH preparation (3.8 mg) from hydroxylapatite chromatography (Fig. 8). The insert shows the electrophoretic pattern obtained by the procedures in refs. 16 and 17.

a. *The Starting Zone*

The concentrated fractions from the preceding chromatographic step were dialyzed against the electrophoresis buffer, diluted 1: 5. The sample volume was about 0.5 ml and in order to minimize the losses of the precious material the dialysis was performed in the following way.

A piece of glass tube (inner diameter about 0.5 cm) was constricted and closed about 1 cm from one end, thereby forming a small chamber with a volume slightly bigger than 0.5 ml. The sample was pipetted into the chamber and this was closed with a dialysis membrane, stretched and held in place by a force-fitting polyethylene ring. The other end of the glass tube was attached to a motor and the tube was rotated (12 rev./min) with the chamber immersed in the diluted buffer. The use of a chamber with a volume only slightly bigger than that of the sample prevented this from being diluted at the development of the Donnan equilibrium. After terminated dialysis the sample could easily be recovered in good yield by the insertion through the dialysis membrane of a constriction pipette.

The sample was applied on top of the gel column by layering under the buffer. A density of the sample exceeding that of the buffer was ensured by the addition of sucrose (6 per cent, w/v).

b. *Pituitary Material*

In each experiment about 4 mg were applied. The electropherogram as obtained from absorption measurements at 230 mμ is given in Fig. 10. The

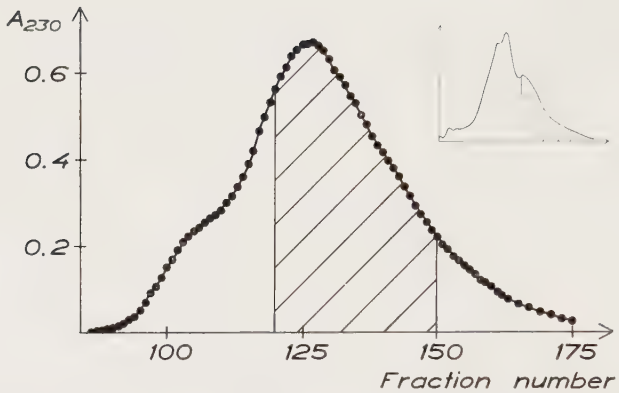

Fig. 11. Polyacrylamide gel electrophoresis of urinary FSH preparation (6.9 mg) obtained by hydroxylapatite chromatography (Fig. 9). The insert illustrates the pattern after application of the procedures in refs. 17 and 64.

results of repeated bio-assays of individual fractions were the determining factor for the selection of the material to be ultimately pooled for characterization. The pooled fractions (those within the hatched area in Fig. 10) were concentrated and assayed. In Table I (p. 33) the value of 14,000 i.u. per mg represents a mean potency of pituitary FSH from three electrophoretic runs.

An overall yield of FSH of about 0.3 mg per 100 g of pituitaries (about 2 μg per pituitary) is provided (Table IV, p. 42).

c. *Urinary Material*

The active material from the three runs on hydroxylapatite was separately concentrated and subjected to electrophoresis in three consequtive runs, about 7 mg being applied each time. One of the electropherograms is shown in Fig. 11; also indicated are the fractions which were pooled and characterized. Assay data and yields from the electrophoresis experiments are summarized in Tables III (p. 38) and V (p. 44). A total of 7 mg, corresponding to 0.85 mg per g[1] of the original concentrate (about 1 μg per liter of urine) was obtained. The average potency of this material was 780 i.u. per mg.

B. Pituitary LH

The major unadsorbed fraction from DEAE-cellulose chromatography of human pituitary concentrates (see Fig. 2, p. 35) was associated with a

[1] From about 8.5 g of urinary concentrate, considering the previous removal of 5–6 % of material (section b, p. 43).

Table VI. Purification of human pituitary LH.

Assay of various fractions for LH activity.

Method of preparation	Fraction	Relative potency: Biological assay: I.U. of HCG/mg[b]	Mean I.U. of HCG/mg	Mean i.u./mg	Radioimmunological assay[a]: i.u./mg	Mean i.u./mg	Index of discrimination [51]
Extraction and ammonium sulphate fractionation (section a:1, pp. 32 ff.)	P 89	11 (7.6–15)			97		
	P 89	9.8 (7.0–13)	10	*63*		100	0.61
	P 90	9.1 (6.0–12)			111		
	P 90	11 (8.4–14)					
DEAE-cellulose chromatography (section a, pp. 34 f.)	D 108	23 (14–41)			190		
	D 108	23 (15–38)	20	*130*		190	0.66
	D 138	16 (7.8–28)			190		
	D 138	19 (13–30)					
SE-Sephadex chromatography (section B, pp. 46 ff.)	SE 23	2000 (1300–3500)			18,000		
	SE 23	1500 (880–2900)	2200	*14,000*		17,000	0.79
	SE 26	2500 (1600–4100)			17,000		
	SE 26	2800 (2100–2200)					

[a] Values from ref. 21.

[b] See footnote *b*, p. 36.

biological LH activity of about 130 i.u. per mg (Table VI). About 50 per cent of the proteins present in such fractions remained in solution after the concentration and dialysis procedures. Most of the original LH activity was recovered in the supernatant and accordingly following dialysis against the acetate buffer at pH 4.35 there was an approximate doubling in LH potency.

1–3 g of dialyzed material were subjected to molecular sieve chromatography on a column of dimensions 7×100 cm. Fractions of about 20 ml were collected and the flow rate was about 2 ml/cm²/h. Depending on the amount of starting material reruns were either performed on the same column under identical conditions or on a narrower column with a proportionally decreased flow rate. The biological LH potency was about 6300 i.u. per mg for material collected from active fractions after two consecutive runs on Sephadex G-100. This represented a one hundred-fold increase in potency over that of the original precursor, which was the pituitary concentrate (see Table VI).

Chromatography on SE-Sephadex resulted in a further two-fold increase

in biological LH potency (Table VI). A major LH-containing fraction was obtained by elution with a buffer concentration of 0.05 *M* (pH 7.0). The final product having a biological LH potency of about 2600 I.U. of HCG per mg (Table VI, fraction SE 26) was obtained following fractionation of 30 mg of hormone preparation on a column of 1.6×15 cm. In this case fractions of 3 ml were collected at a flow rate of 7 ml/cm^2/h.

The two experiments listed in Table VI (fractions SE 23 and SE 26) gave on overall yield of about 1.3 mg purified LH per 100 g of pituitaries. The mean biological LH potency was about 14,000 i.u. per mg. The radioimmunological FSH activity of purified LH was about 4 i.u. per mg, a value less than 0.05 % of the highest value obtained for pituitary FSH (Table I, p. 33).

IV:2. *Hormone Assay*

Tables I, II, III and VI (pp. 33, 36, 38, and 47, respectively) record biological and radioimmunological assay data of various pituitary and urinary fractions prepared as described earlier in the text. In the bio-assays the values for the index of precision (λ) were almost always less than 0.20. This indicates a highly satisfactory precision for both the augmentation and the seminal vesicle assays.

Factors for the conversion of potency estimates from NIH-FSH-S2 units and from HCG units to i.u. of FSH and LH activity, respectively, are provided in Table VII. Factors by which the potency of NIH-FSH-S1 can be expressed in i.u. per mg have been given by Butt *et al.* [26] and by Donini *et al.* [27]. Employing the activity ratio of 1.0/0.87 between NIH-FSH S1 and NIH-FSH-S2 (data supplied by National Institutes of Health) the relevant factor would be 23.1 (Donini *et al.*) and 22.4 (Butt *et al.*) in good agreement with 22.0 (Table VII).

As seen from Tables I and III the index of discrimination[1] [51] is nearly constant in the FSH assays, irrespective of the purity of the fractions. The immunoassay can therefore be used with confidence in a large activity range and as the index of descrimination is close to 1 the potencies are directly comparable with the corresponding biological data. As regards the LH assays there is a certain variation in the index of discrimination. For the fractions involved in the purification of LH (Table VI), the index is, however, fairly constant.

The bio-assay data for pituitary FSH (Table I) indicate that application of the different fractionation methods result in a successive increase in

[1] The ratio of the biological and radioimmunological potencies, both potencies being expressed in i.u. per mg.

Table VII. Conversion factors.

Activity	Method of assay	Reference preparation	No. of assays	Conversion factor[a] (mean potency
FSH	The rat ovarian augmentation assay [20]	NIH–FSH–S2 (1 unit = the activity in 1mg)	6	22.0
LH	The seminal vesicle test [22]	HCG (1 unit = 1. I.U. of HCG activity)	5	6.25

[a] The conversion factor is the number of i.u. equipotent to 1 unit of the reference preparation.

follicle-stimulating potency from 29 to 14,000 i.u. per mg, that is a 480-fold increase in activity. It was possible to increase the biological potency from 8.6 to 780 i.u. per mg by fractionating the urinary concentrate (Table III). This represents a 90-fold increase in activity. Table VI shows an enhancement in biological LH potency from 63 to 14,000 i.u. per mg, or more than a 200-fold increase in potency over the starting material.

Finally, Table II gives information about the intricate question of LH activity in highly purified FSH preparations. The limited supply of these preparations did not allow the performance of bio-assays for LH activity, but the reliance had to be placed upon the sensitive radioimmunological assay. The LH potency of pituitary FSH is 18 i.u. per mg (this value is the mean potency of three preparations). The potency of the most purified pituitary LH (Table VI) is 17,000 i.u. per mg. This latter value must be less than or equal to the potency of pure pituitary LH. It follows that on a radioimmunological basis the content of LH in FSH corresponds to maximally 0.1 per cent by weight. Whether pituitary FSH does contain LH as a contaminant or if the low LH activity is an intrinsic property of the FSH molecule is still an open question. In case the first hypothesis would be the right one, the level of LH is nevertheless too low to be of any significance in the physical and chemical methods utilized for the characterization of FSH.

The radioimmunological LH activity of urinary FSH is 15 i.u. per mg (mean potency of three preparations), a value close to that for pituitary FSH. As no highly purified urinary LH preparation is available, neither from the present work or reported upon elsewhere, the only possible reference material is the purified pituitary LH. With this material as a reference the above considerations for pituitary FSH are also valid for urinary FSH.

IV:3. *Chemical Characterization of FSH*

A. Amino Acid Analysis

Protein samples of 0.25–0.65 mg were hydrolyzed for each analysis.

A:1. *Pituitary FSH*

A total of six analyses were performed; two with 24-h hydrolysates, two with 72-h hydrolysates, one with an oxidized 18-h hydrolysate and one with an alkylated 24-h hydrolysate.

The result is recorded in Table VIII. The alkylated hydrolysate did not contain any S-carboxymethylcysteine and the value obtained for half-cystine was compatible with those obtained with normal hydrolysates. This was considered to prove that no free sulfhydryl groups were present in pituitary FSH.

The coefficient of variation was minimized for $\bar{k} = 143\ \mu\text{moles}^{-1}$, the calculation being based upon the averaged data of lysine, histidine, arginine, glycine, alanine, isoleucine, leucine, tyrosine and phenylalanine. Among these amino acids, histidine is the one which occurs with the lowest number of residues, namely 8 (Table VIII). The cases investigated in the search for the most probable composition were those corresponding to 5–9 residues of histidine per molecule. The figures within the brackets (Table VIII) indicate the number of analyses on which the values are based. The causes for their deviation from six are the following: lysine and histidine, not determined in the oxidized sample, partly destroyed by alkylation; arginine, not determined in the oxidized sample; threonine and serine, values obtained with 24-h and 72-h hydrolysates were extrapolated to zero hydrolysis time by first-order kinetics; proline, too small amounts of hydrolysate in two analyses for an accurate determination; cystine, determined as cysteic acid in the oxidized sample; methionine, decomposed during hydrolysis, therefore only the value obtained with 24-h hydrolysis of the largest amount of sample is given; isoleucine, significantly too low values with 18-h and 24-hydrolysis; tyrosine, destroyed by oxidation; phenylalanine, significantly too low value with 18-h hydrolysis; glucosamine, decomposed[1], only the value from the largest 24-h hydrolysate is considered.

The integral amino acid composition (Table VIII) corresponds to a molecular weight for the protein moiety of pituitary FSH of 29,814. Included in this calculation is the value for tryptophan (see section C, p. 52). The value for glucosamine (Table VIII) is included in Table X (p. 53) and used in the calculation of the molecular weight of the carbohydrate moiety of the hormone.

[1] Recovery 44 % with 24-h hydrolysis, Dr D. Eaker, personal communication.

Table VIII. Amino acid composition of human pituitary and urimary FSH.
(Results are given as means ±S.D. with the numbers of observations in parentheses.)

Amino acid	Pituitary FSH		Urinary FSH	
	Residues according to eqn. (1) (p. 25), $k = 143$	Nearest integer	Residues according to eqn. (1) (p. 25), $k = 75.2$	Nearest integer
Lysine	15.9±0.4 (4)	16	6.8±0.3 (2)	7
Histidine	7.8±0.2 (4)	8	4.0±0 (2)	4
Arginine	10.9±0.3 (5)	11	8.1±0.1 (3)	8
Cysteic acid	24.3 (1)		31.4 (1)	
Aspartic acid	19.3±0.2 (6)	19	27.5±0.3 (4)	27
Methionine sulfone	4.0 (1)		0.32 (1)	
Threonine	26.8 (5)	27	12.1 (3)	12
Serine	19.8 (5)	20	15.9 (3)	16
Glutamic acid	24.8±0.3 (6)	25	20.9±0.4 (4)	21
Proline	16.3±0.7 (4)	16	18.8±0.5 (4)	19
Glycine	14.3±0.3 (6)	14	25.4±0.7 (4)	25
Alanine	14.9±0.3 (6)	15	11.7±0.1 (4)	12
Half-cystine	25.9±2.7 (5)	26	31.6±1.9 (3)	32
Valine	17.7±0.5 (6)	18	11.3 (1)	11
Methionine	4.0 (1)	4	0.51 (1)	1
Isoleucine	9.6±0.1 (3)	10	5.0 (1)	5
Leucine	12.3±0.2 (6)	12	10.8±0.2 (4)	11
Tyrosine	13.1±0.4 (5)	13	4.0 (2)	4
Phenylalanine	9.2±0.2 (5)	9	7.0±0.4 (3)	7
Tryptophan	3.6	4	2.0	2
Glucosamine	29.7 (1)	30	7.7 (1)	8
Total[a]		254		208

[a] Half-cystine is included as 13 (pituitary FSH) and 16 (urinary FSH) cystine residues; glucosamine not included.

A:2. *Urinary FSH*

Four analyses of urinary FSH were performed; one with each of the four types of hydrolysates employed for pituitary FSH.

The amino acid composition of urinary FSH is recorded in Table VIII. Also in urinary FSH no free sulfhydryl groups could be detected.

Given within brackets are the number of analyses on which the values in the table are based. The reasons for these numbers being lower than four are the same as given for pituitary FSH. It should be added that for valine only the value obtained with 72-h hydrolysis was considered, other values being significantly lower. The tyrosine values were corrected, considering the decomposition as a first-order reaction. The coefficient of variation

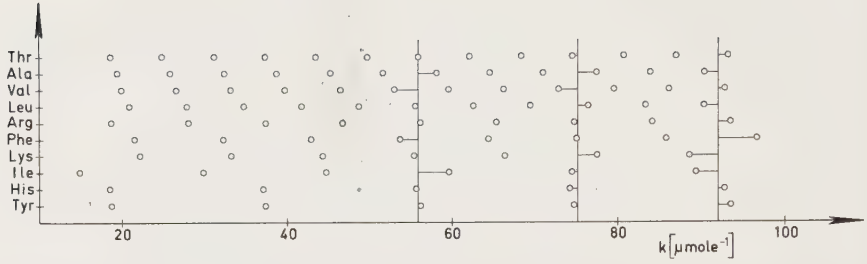

Fig. 12. Plot used for the calculation of the molecular weight of the protein moiety of urinary FSH from amino acid analysis data (see the text).

had a minimum for $\bar{k} = 75.2$ μmoles ^{1}, when the calculation was based upon the values determined for those amino acids given in Fig. 12. As indicated in the figure the probability for three different compositions of urinary FSH (corresponding to $\bar{k} = 55.9$, 75.2 and 92.1 μmoles^{-1}) were investigated.

A molecular weight of 23,600 for the protein moiety of urinary FSH was calculated from the composition given in Table VIII.

B. Molar Extinction Coefficient

Insertion in eqn. (8) (p. 28) of data obtained for four different samples of pituitary FSH, analyzed according to Spackman *et al.* [31], gave $\varepsilon =$ 35,500 mol^{-1} 1 cm^{-1}. For urinary FSH insertion of values for two samples, resulted in $\varepsilon = 17{,}400$ mol^{-1} 1 cm^{-1}.

C. Estimation of Tryptophan

The results of the tryptophan estimation, calculated from eqn. (9) (p. 28) by insertion of the various parameters, are given in Table IX. Given in the table are also some relevant data for pituitary and urinary FSH.

Table IX. Tryptophan contents of human pituitary and urinary FSH.

Preparation	ε mol^{-1} 1 cm ^{1}	Residues per mole			
		Tyr	Cys	Try calculated	Try nearest integer
Pituitary FSH	35,500	13	13	3.6	4
Urinary FSH	17,400	4	16	2.0	2

Table X. Carbohydrate components of human pituitary and urinary FSH. (Results are given as means ± S.D. with the numbers of observations in parentheses.)

	Pituitary FSH			Urinary FSH		
		Residues per mole			Residues per mole	
Sugar	Content %	Calculated	Nearest integer	Content %	Calculated	Nearest integer
Hexose	19.6±0.1 (2)	38.7	39	14.5±0.1 (2)	14.0	14
Glucosamine			30			8
Sialic acid	7±1 (3)	8.0	8	3.1±0.1 (3)	1.75	2

D. Carbohydrate Analysis

The results of the hexose determination performed on duplicate samples of the two hormone preparations are given in Table X. Also listed in this table are the sialic acid analysis data from triplicate samples. The amounts of samples analyzed by each of the two procedures were 0.1–0.2 mg for a single determination. The data are expressed as per cent by weight of protein (as determined spectrophotometrically at pH 7.0) and also as the number of residues per mole of FSH. These numbers were obtained by multiplication of the percentage values by the expression $\varepsilon/(100 \cdot M)$, where ε is the relevant molecular extinction coefficient (section B, p. 52) as determined in connection with the amino acid analysis and M is the molecular weight of hexose (180) or of N-acetylneuraminic acid (309). Finally Table X also contains the number of glucosamine residues as determined by the amino acid analysis procedure (section A, pp. 50 ff.).

From the values given in Table X, the molecular weights of the carbohydrate moieties of pituitary and urinary FSH were calculated to be 13,487 and 4,142, respectively.

E. Partial Specific Volume

Insertion in eqn. (10) (p. 29) of the values from Tables VIII (p. 51), IX (p. 52), and X (p. 53) gave $\bar{v} - 0.69$ g^{-1} cm^3 for both pituitary and urinary FSH.

IV:4. *Physical Characterization of FSH*

A. Sedimentation Constant

The sedimentation velocity experiments were performed in a 2°, 12 mm double sector cell at 5.0°C and at a speed of 59,780 rev. per min. The buffer

Table XI. Sedimentation analysis of human pituitary and urinary FSH at 5°C.

Preparation	Protein mg/ml	s_5 S	$s_{20,w}$ S
Pituitary FSH	5.6	1.90	2.95
	4.4	1.92	2.98
	3.1	1.91	2.96
	Infinite dilution		2.96
Urinary FSH	5.8	1.26	1.96
	4.8	1.29	2.00
	3.3	1.33	2.07
	Infinite dilution		2.21

solution used in all runs had the composition 0.01 *M* sodium phosphate, pH 7.0, and 0.10 *M* sodium chloride. The samples were prior to centrifugation extensively dialyzed against this buffer (in the manner described earlier, section a, p. 45). The sample volume added to the cell was about 0.3 ml and the concentration range studied was 6–3 mg per ml.

The results from three experiments on each of pituitary and urinary FSH are recorded in Table XI. The observed sedimentation coefficients (s_t) were converted to $s_{20,w}$ by the equation

$$s_{20,w} = s_t \cdot \frac{\eta_t}{\eta_{20,w}} \cdot \frac{1 - \bar{v}_{20}\varrho_{20,w}}{1 - \bar{v}_t\varrho_t}, \tag{12}$$

where the symbols have their generally accepted significance [42, 43]. The value for the partial specific volume ($\bar{v}$) was that calculated from the amino acid and carbohydrate analyses (section E, p. 53). Because of the inherent uncertainty in such a calculation [41] no correction of the $\bar{v}$-value for temperature dependence was attempted.

The sedimentation coefficient for pituitary FSH did not show any significant concentration dependence in the range studied (Table XI), and the value for $s^0_{20,w}$ is the mean from the three experiments. For urinary FSH, $s^0_{20,w}$ was calculated by the method of least squares.

Depending on the very limited supply of material the lower concentrations were prepared by successive dilutions of material recovered from the centrifugation cell. Representative photographs from the sedimentation velocity experiments are shown in Fig. 13 (pituitary FSH) and Fig. 14 (urinary FSH). Both patterns show only one sedimenting boundary, recorded as a single symmetrical peak.

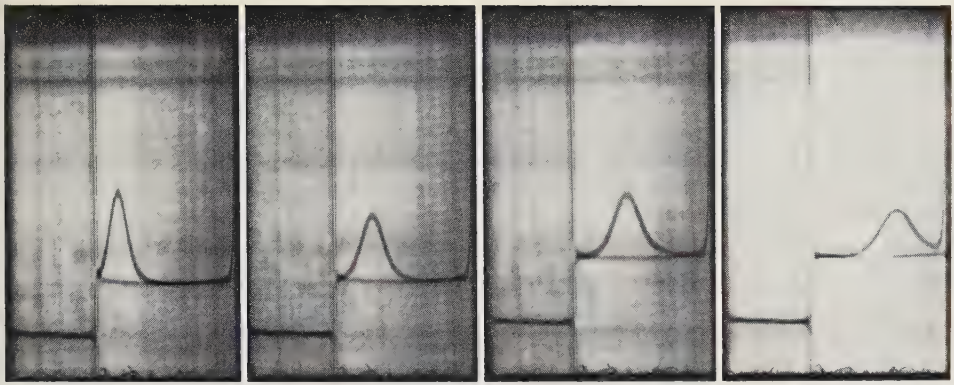

Fig. 13. Sedimentation patterns of pituitary FSH at 59,780 rev./min and a protein concentration of 5.6 mg per ml. Pictures were taken 47 min, 79 min, 111 min and 175 min after the centrifuge was up to speed.

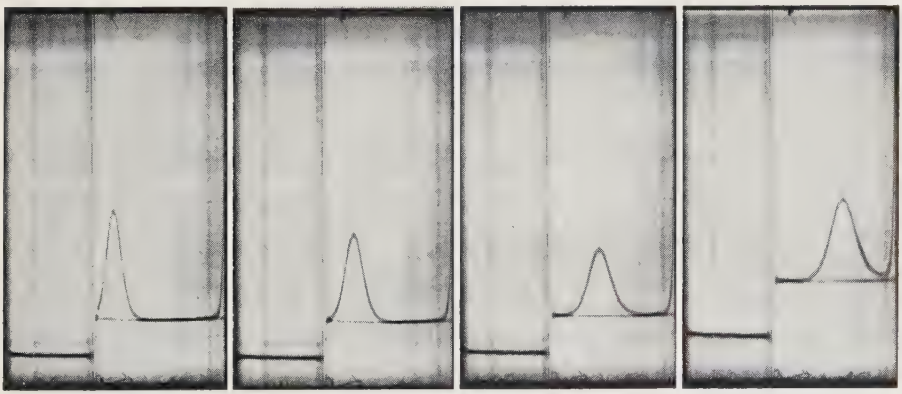

Fig. 14. Sedimentation patterns of urinary FSH at 59,780 rev./min and a protein concentration of 5.8 mg per ml. Pictures were taken 58 min, 90 min, 154 min and 218 min after the centrifuge was up to speed.

B. Molecular Weight

The sedimentation equilibrium experiments were performed in the same solvent as utilized in the sedimentation velocity runs. The sample volume used in the double sector cell was 0.15 ml. The centrifuge was operated at 10.587 rev. per min and the temperature was 5.0°C. To assure the attainment of equilibrium the duration of the runs were about 60 h. For the concentration determination in the synthetic boundary cell (2°, 12 mm) 0.1 ml of sample was utilized. The sample compartment also contained 0.05 ml of silicone oil.

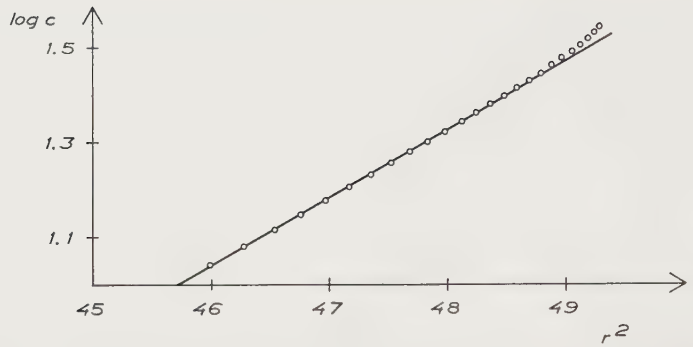

Fig. 15. Analysis of pituitary FSH by sedimentation equilibrium. The logarithm of the concentration (in fringes) is plotted as a function of the square of the radial distance (in cm^2).

In Fig. 15 the plot from a run of pituitary FSH is presented. The original sample concentration was 0.38 per cent (spectrophotometric determination). The slight deviation from a straight line relationship in the last 15 per cent of the plot are likely to reflect some molecular interaction caused by the increased protein concentration in the bottom region of the cell. From the slope of the straight line (Fig. 15) the apparent molecular weight of pituitary FSH was calculated (eqn. (11), p. 30) to be 41,000 ($\bar{v} = 0.69$ g^{-1} cm^3, section E, p. 53).

As the sedimentation coefficient for pituitary FSH was not significantly concentration dependent in the range 0.3–0.6 % (Table XI) and as the diffusion coefficient for proteins exhibits a less concentration dependence than is observed for sedimentation coefficients [52], the value of 41,000 was considered as a good approximation of the true molecular weight of the hormone. (These considerations are based upon the Svedberg equation in its original form [42, 43]; the true molecular weight being proportional to $s^0_{20,w}/D^0_{20,w}$).

Fig. 16 is a plot from an experiment with urinary FSH (protein concentration 0.46 %). The straight line connecting the points was calculated by the method of least squares. The data provided a value of 26,700 for the molecular weight of the hormone. The sedimentation coefficient for urinary FSH is at a protein concentration of 0.46 % about 9 % lower than at zero concentration (Table XI). If as an approximation, the variation in the diffusion coefficient is considered as negligible when compared with that in the sedimentation coefficient, the true molecular weight of urinary FSH would be 29,400. The ultracentrifuge studies would thus be compatible with a molecular weight of urinary FSH, being in the range of 26,700–29,400.

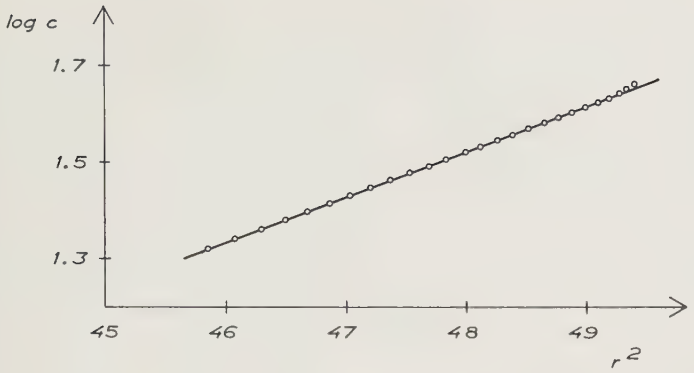

Fig. 16. Analysis of urinary FSH by sedimentation equilibrium. The logarithm of the concentration (in fringes) is plotted as a function of the square of the radial distance (in cm^2).

C. Free Zone Electrophoresis

The electrophoresis was performed in 0.1 *M* Tris-HCl buffer, pH 8.7 (pH determined at 20°C). The current was 3 mA and the temperature of the cooling water was maintained at 2.3°C. Preliminary experiments had indicated similar electrophoretic mobilities for pituitary and urinary FSH. In order to establish if there was a significantly measurable difference, the two preparations were introduced into the revolving tube as two separate zones and run simultaneously. 40 μg of pituitary FSH and 30 μg of urinary FSH were applied, each hormone in a volume of 10 μl of the electrophoresis buffer.

The positions of the starting zones in the rotating quartz tube are indicated in Fig. 17; the distance in the tube between the two application sites was 5.5 cm. The electrophoretic migration was followed by scanning the electrophoresis tube repeatedly for ultraviolet absorbing material (Fig. 17). Both pituitary and urinary FSH were recorded as single symmetrical peaks throughout the course of the experiment. From the experimental data the mobilities of the two hormones were calculated as described in detail by Hjertén [45]. The values obtained were for pituitary FSH $-5.83 \cdot 10^{-5}$ cm^2 sec^{-1} $volt^{-1}$ and for urinary FSH $-6.17 \cdot 10^{-5}$ cm^2 sec^{-1} $volt^{-1}$, and refer to the Tris-HCl buffer at 0°C. It follows that the difference in mobility is 6 per cent.

At the end of the run fractions were withdrawn and assayed for FSH activity. Full evidence was obtained that the activity was associated with the material corresponding to the recorded peaks.

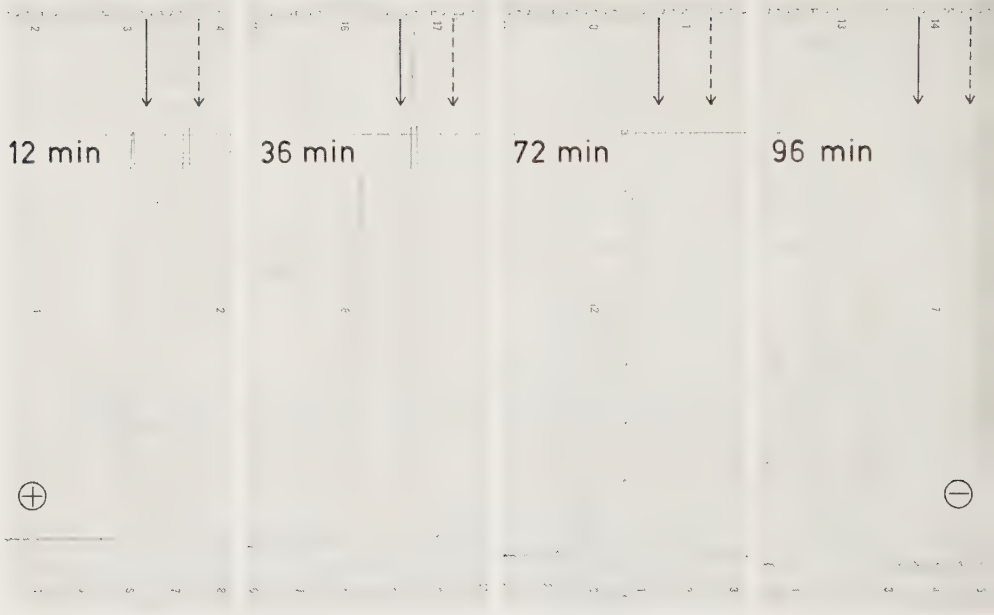

Fig. 17. Ultraviolet recording from free zone electrophoresis of pituitary and urinary FSH. The arrows indicate the positions of the starting zones, the dashed arrow represents the position of pituitary FSH.

D. Ultraviolet Absorption Spectrum

Examination of the spectrum of pituitary FSH in 0.01 *M* potassium phosphate, pH 7.0, indicated a maximum at 277 mμ and a minimum at 251 mμ. Urinary FSH, studied in the same buffer, showed a maximum at 276 mμ and a minimum at 255 mμ. These spectra are shown in Fig. 18. The appearance of the spectra compared with those of the free amino acids [35] are indicative of a higher ratio of tryptophan to tyrosine in urinary FSH than in pituitary FSH. The estimation of tryptophan in FSH (section C, p. 52) confirmed this hypothesis.

In 0.1 *N* NaOH–8 *M* urea, a medium suggested by Wetlaufer [35], the maximum observed for pituitary FSH was shifted to 291 mμ and the minimum to 276 mμ. Urinary FSH had in this medium no well-defined maximum or minimum. A point of inflection indicative of tryptophan could be observed at 289 mμ. Spectra of both hormones showed a time dependence in alkali in the interval studied which was from 15 min to 16 h after preparation of the solutions. Correction for non-specific absorption (linear extrapolation from the values at 340, 350 and 360 mμ) indicated that pituitary

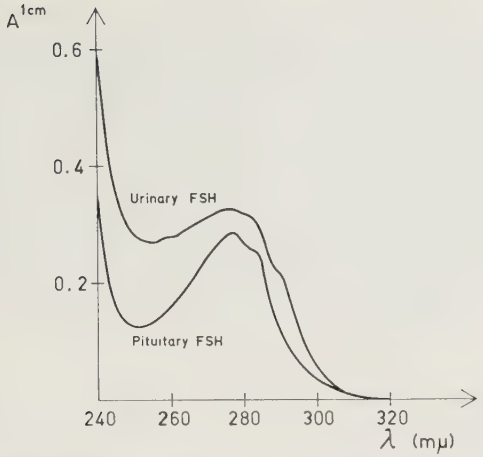

Fig. 18. Ultraviolet absorption spectra of pituitary and urinary FSH in 0.01 *M* potassium phosphate buffer, pH 7.0.

FSH reached its highest absorptivity at 280 and 305 mμ after 1 h; at 290 and 295 mμ the values at 15 min were the greatest. These two values were unchanged after 1 h but thereafter there was a decrease in absorbance in the whole range of 280–305 mμ. The spectrum of urinary FSH showed the highest values at the above wavelengths 2 h after the solution was prepared.

IV:5. *Immunological Studies of FSH*

A. Neutralization of the Biological Effect of FSH

It should be noted that the pituitary FSH preparation injected into the guinea pigs was only about 50 per cent FSH (as judged from bio-assay results). In order to investigate whether the antiserum obtained was capable of neutralizing the biological effect of FSH, as measured by the ovarian augmentation assay, the following procedure was employed.

Increasing quantities of antiserum were added to a number of test tubes, each of which contained the same amount of pituitary FSH in 0.01 *M* phosphate buffer, pH 7.0. The samples were incubated for 1 h at 37° and then for 24 h at 4°C. After centrifugation, HCG was added to the supernatants and these were biologically assayed (40 IU of HCG per animal). An incubated control (no antiserum) gave an adequate response but all the other samples were negative. It was observed, however, that with increasing amounts of antiserum there was a parallel decrease in the ovarian weights; with the highest amounts of antiserum the ovarian weights were significantly

lower than those obtained when HCG alone was administered. This was taken to indicate that the antiserum contained antibodies reacting with HCG. As the response in the augmentation assay is based upon the synergistic effect of FSH and HCG it follows that no conclusions can be reached about whether or not the serum contained any antibodies reacting with FSH. (In a later experiment it was found that a sample of HCG, which had been incubated with antiserum, gave rise to lower ovarian weights than an incubated HCG control.)

To obtain unequivocal proof of the presence in the serum of antibodies directed against FSH the following procedure was adopted. A column tube (1.7 × 45 cm) was packed with Sephadex G-150 in 0.1 *M* Tris-HCl buffer, pH 8.0, also contaning 0.3 *M* NaCl. Twenty μl of the antiserum diluted to 400 μl with the buffer, were applied to the column. Fractions of about 1 ml were collected at a flow rate of 5 ml per h. The effluent profile (readings at 230 mμ) was as expected [53], with three partly separated peaks. The fractions corresponding to the first two peaks were pooled and so also were the fractions corresponding to the third peak. The two pooled fractions and a buffer control were incubated with an appropriate amount of pituitary FSH (about 6 μg containing about 25 i.u. of FSH activity) for 1 h at 37° and 24 h at 4°. Following centrifugation and addition of HCG, the three samples were assayed (40 IU of HCG were administered to each animal). The sample corresponding to the first two peaks in the chromatogram was negative whereas the activity of the sample corresponding to the third peak did not deviate significantly from that of the control.

This experiment thus provided the expected information that the antibodies studied were eluted in the first of the pooled fractions. It follows that the volume of this fraction added to the void volume of the column can be considered as the maximal elution volume (Ve^{max}) of antibodies from the column used. The value of Ve^{max} is of the utmost importance in the subsequent experiments.

Two equal samples of pituitary FSH were prepared in the Tris-HCl buffer. Each sample contained as before about 25 i.u. of FSH activity. To one of the samples was added 20 μl of the antiserum, to the other 1.4 mg of bovine albumin. The amount of albumin was chosen to give approximately the same amount of protein in the two samples. The sample volumes were 0.5 ml and both samples were incubated under the conditions described above. Both samples were then separately chromatographed on the G-150 column and in both cases those fractions were collected (17 ml), which followed immediately after the established volume Ve^{max}. HCG was added as before and the two eluates were assayed. The potency of the eluate derived from the sample incubated with the antiserum was in the range found

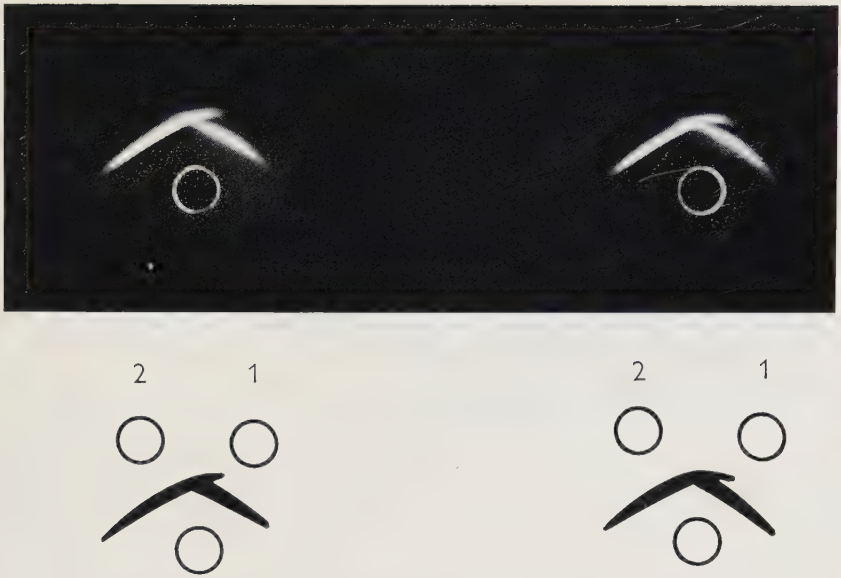

Fig. 19. Gel diffusion analysis of pituitary and urinary FSH using an antiserum to a pituitary FSH preparation. Well 1 contained urinary FSH, well 2 pituitary FSH. The ratio of the amounts of urinary FSH to pituitary FSH was in the left system about 2:1, in the right system about 3:1.

when assyaing only HCG (40 IU per animal). In contrast, the other sample had retained its full activity. These findings proved that the serum could inhibit completely the biological effect of pituitary FSH.

Repetition of the experiments with urinary FSH instead of pituitary FSH provided the same results, thus indicating a cross-reaction between the two FSH preparations.

B. Gel diffussion

The thickness of the agarose layer used in the gel diffusion experiments was about 2 mm and the distance between the symmetrically placed antibody well and the antigen wells was 3.2 mm. The volume of a well was about 5 μl. Prior to use the antiserum was concentrated about three times by ultrafiltration, nevertheless 2–3 soakings of the central well were necessary to get precipitin lines that could easily be detected. In experiments with this concentrated antiserum urinary FSH (Fig. 19, well 1) gave a reaction of partial identity with pituitary FSH (well 2). In the left system (Fig. 19) the ratio of the amounts of urinary FSH to pituitary FSH was about 2:1; in the right system this ratio was increased to about 3:1, the amount of anti-

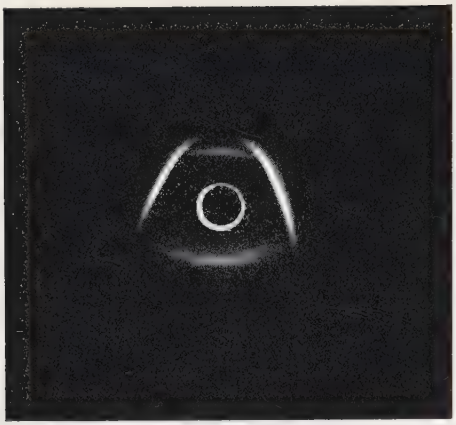

Fig. 20. Gel diffusion analysis of pituitary and urinary FSH using an antiserum to a pituitary FSH preparation. Wells 1 and 3 contained pituitary FSH, wells 2 and 5 urinary FSH; wells 4 and 6 were empty. The ratio of the applied amounts of urinary FSH to pituitary FSH was 3 : 1.

serum being equal in the two systems. At increased amounts of urinary FSH (right system) the spur produced by the extra determinants in pituitary FSH was deviated by the urinary sample. This would suggest the possible presence in the urinary sample of some material identical with pituitary FSH.

To study further this possibility an experiment was performed designed as shown in Fig. 20. Again the ratio of urinary and pituitary FSH was 3 : 1. As in earlier experiments pituitary FSH gave one precipitin line (wells 1 and 3) against the pituitary anti-FSH, giving strong support for its homogeneity. With urinary FSH in well 2, the partial identity of the two hormones was confirmed. In view of no detectable split of the urinary FSH emanating from well 5 (wells 4 and 6 being empty), the slight deviation of the spur (wells 1 and 3) cannot for the present be attributed any significance.

The antiserum used in these diffusion experiments contained antibodies reacting with HCG (section A, pp. 59 ff.). The antigen used for immunization was not pure with respect to pituitary FSH and contained up to 50 per cent of contaminating material. In particular luteinizing hormone may have been present in the antigen and may have stimulated the formation of these cross-reacting antibodies. However, in gel diffusion, a highly purified pituitary LH preparation gave no precipitin line with the antiserum. This result makes it unlikely that the precipitin lines shown in Figs. 19 and 20 were due to LH antigen present in the FSH preparations.

V. DISCUSSION

V:1. *Purification*

A. Pituitary and Urinary FSH

A:1. Extraction and Ammonium Sulphate Fractionation

a. *Pituitary Material*

a:1. *Selection of starting material*

To obtain reproducible results in fractionation work it is necessary that the relevant properties of the starting material are the same from time to time. This is never strictly the case in work with pooled material of biological origin. By careful selection it is possible, however, to minimize the unavoidable variations in the compositon of the starting material.

Work on human pituitaries has included three different groups of glands, namely frozen, acetone-preserved and lyophilized glands. As mentioned earlier the two first of these categories have been considered in this study.

Frozen glands were used to prepare FSH for physical, chemical and biological characterization. The reason were that frozen glands are the most likely to contain FSH in its native form and therefore also to give the most reliable parameters when characterized.

In principle, FSH for chemical characterization should be prepared from all three groups of glands. Conformational changes which may be inherent in operations such as lyophilization and acetone treatment are not relevant in this connection. In practice, however, there are reasons why FSH prepared from pretreated glands is less preferable for chemical characterization. In work with pituitaries collected at many different hospitals it is not possible to control unwanted handling of the glands prior to their delivery. From the appearance of a batch of frozen pituitaries it is immediately evident whether the glands have been thawed and then frozen again (such batches may be used for clinical purposes, see below). A corresponding knowledge of the history of acteone-treated or lyophilized glands cannot be acquired by visual inspection. One cannot, for example, see if the latter, prior to lyophilization, have been repeatedly frozen and thawed or if the former have been stored at room temperature for a long period of time. With such uncontrolled conditions in mind, it seems justified not to overlook the possibility of the occurrence in pretreated glands of enzymatically degraded FSH. (In the earlier stages of this study, starting with lyophilized

glands, two electrophoretically separated forms of FSH were obtained. The reasons for their existence were not established.)

Expressed in activity units, the yield from frozen glands were always higher than from acetone preserved pituitaries (section a, pp. 32 ff.). Frozen glands are therefore preferable also when preparing clinical grade material. However, as it is the biological activity which is the determining factor in the clinical utilization, and as alterations in the native structure are not critical, frozen glands, which had accidently been thawed and acetone-preserved glands were also employed as starting material (see section VI, pp. 86 f.).

a:2. *Choice of buffer and pH value*

The extraction conditions for the homogenized glands were chosen under the assumption that the human pituitary contains proteinases closely related to those found in the pig pituitary [54]. The presence of two enzymes was reported; one of them was inhibited by phosphate ions, and the total proteinase activity had a minimum around pH 6. After the present extraction procedure for human pituitary FSH had been developed and found to give better yields than reported elsewhere, further papers about proteolytic activities in pituitary extracts have been published. Ellis [55], working with sheep glands, suggested the presence in crude extracts of at least eight different proteolytic enzymes and Reichert and Parlow [56] and Lewis [57] have reported on proteolytic activity in human hormone preparations. A check by the Anson procedure [58] for proteolytic activity in extracts A, B and C prepared from frozen glands (section a:1, pp. 15 f.) gave negative results. (The possibility that this might reflect a different ease of extraction of the enzymes from frozen glands and from pretreated glands, as used by others, was not investigated.) These findings in combination with the good yield of the crude extract and the ultimate isolation of only one protein associated with follicle-stimulating activity are the motives for retaining the extraction conditions unchanged.

a:3. *Fractionation with* $(NH_4)_2SO_4$

In work dealing with pituitary hormones $(NH_4)_2SO_4$ has been used as a protein-precipitating agent for about 35 years. The finding that FSH, unlike many other proteins, was soluble in half-saturated $(NH_4)_2SO_4$ solution dates from 1940 [59]. The concentration range used in the present study for precipitating the gonadotropins was established by a stepwise increase in the $(NH_4)_2SO_4$ concentration from 50 % to 90 %. Each step entailed an increase of 5 %, and the precipitates which formed were collected by centrifugation and assayed for FSH activity. Significant activities were found in

the range 55–70 % saturation (the added amount of 175 g salt per liter solution corresponds to a slightly higher value of the final $(NH_4)_2SO_4$ concentration). This is true for frozen glands; in work with acetone-treated glands, a further addition of 50 g of the salt per liter solution was needed for the complete precipitation of FSH.

a:4. *Time for a preparation*

A further approach in the attempts to obtain a high yield of FSH was to compress the time schedule. Before the routine application of the comparatively short times used for the extraction and precipitation procedures, controls were performed in the following way. After the third washing of the homogenized pituitaries was terminated, the residues were again suspended in buffer and extracted with stirring overnight. Subsequent centrifugation and ammonium sulphate fractionation gave only negligible amounts of material both in the GH fraction and in the gonadotropin fraction. It is therefore evident that a prolonged extraction does not increase the yield of hormones.

To check whether the two precipitations with $(NH_4)_2SO_4$ were complete extracts B and C were left at 4° overnight. Subsequent centrifugations did not reveal the presence of material in amounts worth saving.

A further shortening of these steps was not attempted.

a:5. *Dialysis*

Material prepared from frozen glands and intended for clinical use was in earlier experiments dialyzed against distilled water. A precipitate was formed if the dialysis was continued long enough to make the content of $(NH_4)_2SO_4$ negligible. The precipitate was collected by centrifugation and washed with small amounts of water. Its solubility in buffers at pH values around neutrality was low and even in diluted alkali solution it was only slightly soluble. An extract of the precipitate at pH 7 was assayed in relevant doses but did not show any FSH activity.

In contrast, only very small quantities of precipitate were observed following dialysis against phosphate buffer at pH 7 (of extracts intended for further fractionation). It was also found that the specific activities of the supernatants were approximately the same, irrespective of whether the dialyses were performed against this buffer or water. The conclusion was that the precipitate formed by dialysis against water either contained FSH and inert proteins to similar extents or consisted only of inert proteins, leaving all the FSH in solution but in a partly denaturated and therefore less active form. The losses were estimated to be about 40 %, and on account of the scarity of material it seemed important to improve the yield of the

clinical product. This was possible by performing the dialysis against the volatile buffer 0.001 *M* NH_4HCO_3; a minimal amount of precipitate was formed and a salt-free product was provided after lyophilization of the supernatant.

A:2. DEAE-Cellulose Chromatography

a. *Pituitary Material*

DEAE-cellulose column chromatography was already utilized as a fractionation tool in the first report about this research [60]. During attempts to increase the effectiveness of the methods used, the experimental conditions for the DEAE-cellulose step were revised. Using potassium phosphate buffer (pH 7.0) as an eluent it was found that equilibration at a concentration of 0.02 *M* resulted in a maximal amount of material being excluded from the column including virtually all the LH. In contrast all the FSH remained adsorbed onto the column. Following equilibration with 0.03 *M* buffer most of the FSH activity was in the unadsorbed fraction and if a concentration of 0.04 *M* was used all the FSH passed directly through the column. Thus the use of starting buffers of a higher concentration than 0.02 *M* are not advantageous in the separation of LH and FSH.

Before the introduction of the increase in buffer concentration from 0.02 *M* to 0.06 *M* for the elution of FSH the effect of smaller increases was investigated. It was found that even an increase to 0.04 *M* buffer eluted virtually all the hormone activity. The corresponding peak in the chromatogram was, however, broad and poorly defined when compared with the sharp peak obtained with the 0.06 *M* buffer. Furthermore, in the subsequent step (molecular sieve chromatography, section A:3, pp. 17 f.) material eluted at the 0.06 *M* level provided a more distinct effluent profile in the relevant area.

The reason for the decrease of flow rate when eluting with the 0.06 *M* buffer was essentially a practical one. With a flow rate of 5 ml/cm²/h a fraction collector equipped with a 60 tubes turn-table could be left overnight without attention. Fraction volumes of 10–12 ml were used in order to detect even the fine structure of the chromatogram.

The conditions ultimately employed gave an FSH fraction with a 10-fold increase in potency over that of the clinical grade material. To obtain this degree of purity only fractions of high relative potencies were included. This necessarily implied losses of active material. In the present situation active fractions at the rear edge of the first peak eluted by the 0.06 *M* buffer (Fig. 2, p. 35) were discarded. This loss had to be balanced against the attainable advantages in the subsequent purification, and evaluated in the light of practical experience.

b. *Urinary Material*

The supply of urinary material was less than the supply of pituitaries. Pituitary material was therefore generally used when the optimal conditions for the application of a certain method were being established. These conditions were then utilized for fractionation of the urinary material.

Rechromatography of the material corresponding to the minor peaks eluted by the 0.02 *M* buffer (Fig. 3, p. 37) still resulted in material with FSH activity not being adsorbed to the column. This suggested the presence in the original concentrate of more than one active form of FSH such as different metabolites. Being minor components this material was not considered.

A:3. Molecular Sieve Chromatography

By utilizing two consecutive runs on Sephadex G-100 the potencies of the pituitary and urinary materials were increased by factors of about 20 and 3, respectively.

In the earlier stages of this work the denser gel Sephadex G-75 was also tried. However, the resolution obtained in the relevant area was inferior to that obtained using G-100.

The importance of a critical selection of fractions for further work was discussed in connection with the fractionation of pituitary material on DEAE-cellulose (section a, p. 66). If unsuitable fractions were included (that is, if the hatched area in Fig. 2 was extended), an extra peak was obtained in the chromatogram. The position of the maximum of this peak was fractionally to the left of the hatched area of Fig. 4. In some early experiments this extra peak was of a dominating size, resulting in a decrease of the effectiveness of the molecular sieving procedure.

A:4. Hydroxylapatite Chromatography

Chromatography on hydroxylapatite led to an approximate doubling of the potencies of both the pituitary and urinary FSH.

The choice of the pH of the buffer was determined by the observation that free zone electrophoresis at pH 7.0 of a sample of a pituitary FSH preparation resulted in a sudden formation of a precipitate. (The material subjected to this electrophoresis was purified as described in sections A:1–A:3, pp. 15 ff.) One possible interpretation of the observed phenomenon (still unexplored) is that pituitary FSH became labile at this pH when some protective substances were electrophoretically removed. At pH 8.6 no precipitate was formed. Assumming that this interpretation is correct and that protective substances might be separated from FSH also by the chroma-

tographic procedure, this was performed at pH 7.8. It is evident from the above that the pH value should have been even higher but as the capacity of the adsorbent decreases with increasing pH [61], the value of 7.8 was chosen as a compromise.

Before the stepwise elution procedure was finally established, a variety of eluent concentrations were investigated. It was found for instance, that equilibration with 0.001 *M* buffer followed by 0.003 *M* buffer as eluent provided part of the FSH activity in this step, the remainder being eluted with 0.01 *M* buffer. As mentioned above, no significant amounts of activity emerged from the column using 0.001 *M* buffer as starting buffer. This concentration was therefore established as the highest to be employed with confidence for binding all the activity to the adsorbent.

If the starting buffer was 0.01 *M* all FSH activity was eluted directly. In principle it is advantageous to select conditions such that the substance to be isolated is not retarded on the column. Under such circumstances the total recovery of the substance is optimal and the risk of the substance undergoing alterations during the adsorption-desorption processes is eliminated. However, other aspects must also be considered. The subsequent purification step was zone electrophoresis (section A : 5, pp. 18 ff.) and it was found that the direct use of 0.01 *M* buffer provided a material less suitable for the ultimate isolation of FSH than was the product obtained by adsorption–desorption (0.001 *M*–0.01 *M*).

The 0.03 *M* step was of no particular importance and may be incorporated in the 0.5 *M* step. The reason for using 0.03 *M* buffer was that assays of the eluted material provided an extra control for the reproducibility of the chromatographic procedure.

A : 5. Polyacrylamide Gel Electrophoresis

a. *Pilot Experiments*

The working out of the electrophoretic step was very time-consuming, primarily becuase of the small amount of material available. Methods utilizing various supporting media were tried under different experimental conditions and all these pilot studies had to be performed on a micro scale. Pituitary material and, to a less extent, also urinary material prepared as described by DEAE-cellulose chromatography and two consecutive runs on Sephadex G-100 (sections A : 1–A : 3, pp. 15 ff.), was subjected to electrophoresis in columns stabilized by cellulose powder, by suspensions of agarose or by polyacrylamide gel. The columns utilized in the two first methods were in principle constructed as described by Porath *et al.* [62] and by Hjertén [63], respectively. The apparatus for the gel electrophoresis was a

simplified version of that shown in Fig. 1 (p. 19), consisting of a glass tube equipped with a simplified elution chamber. All column tubes utilized for these pilot runs had inner diameters between 0.5 and 0.25 cm and amounts down to 0.5 mg were fractionated. The protein distribution was estimated using microcuvettes; either directly at 230 mμ or by the Folin-Lowry reagent at 750 mμ. Buffers with pH values between 5.0 and 9.7 and having a range of molar concentrations were tried. Worth mentioning in this connection is the observation of precipitates being formed at pH 5. This phenomenon (detectable in the transparent agarose columns) seems similar to what was previously mentioned about precipitation during runs in the free-zone electrophoresis apparatus (section A:4, pp. 67 f.).

The reasons for giving the preference to polyacrylamide gel electrophoresis were, that this method provided the best resolution in combination with a good reproducibility. A further advantage with the method is that any kinds of aggregates which might be formed in runs of long duration, will be effectively retarded (owing to the sieving properties of the gel) and their influence on the resolution will thus be eliminated.

b. *Runs in Polyacrylamide Gels*

Based upon experience from the pilot studies all preparative runs in the apparatus described (Fig. 1, p. 19) were performed in Tris-HCl buffer at pH 8.7. In previous reports [16, 17, 64] (before the introduction of the hydroxylapatite step) pituitary and urinary FSH were isloated from gels of the composition T = 5.13 %, C = 2.53 % (0.2 *M* buffer) and T = 3.07 %, C = 2.53 % (0.05 *M* buffer), respectively.

Before the establishment of the experimental conditions used in the present investigation (T = 5.13 %, C = 2.53 % and 0.05 *M* buffer) further experiments were performed both on a preparative and on an analytical scale. The total concentration T was varied from 3.07 % to 7.70 % (C being kept constant at 2.53 %) and buffers of concentrations between 0.003 *M* and 0.2 *M* were tried. Certain difficulties to polymerize reproducibly the gels with T = 3.07 %, balanced against the increased electrophoretic retardation in the denser gels are the motives for the choice of T = 5.13 %.

The lowest buffer concentrations provided the broadest zones, probably depending on too large a difference in conductivity between the zones and the surrounding medium. With the highest buffer concentration on the other hand the time for completion of a run became unsatisfactory long. The 0.05 *M* buffer was considered as a good compromise.

In the following a few construction details of the electrophoresis apparatus (Fig. 1) and some factors of importance for the separations will be discussed.

The shaping of the apparatus with a central cooler provides a gel layer with a thickness of only 0.65 cm at a cross section of about 3 cm^2. Hereby a more efficient dissipation of the joule heat is obtained and runs can be performed in this column at an essentially higher current than that, which can be utilized in a column with the same cross section, but without inner cooling.

The water flow through the tube C was regulated in such a way that the temperature of the water from the outlet was equal to that of the buffer in the electrode vessel V2. The maximal current which could be used without getting parabolically shaped zones was estimated by the model substances bromphenol blue and albumin (stained with this dye). A high current involves a shortening of the duration of a run and consequently a smaller risk for denaturation of labile substances.

The requirement as to a good elution chamber is that a moderate rate of the buffer flow guarantees an effective washing out of substances emerging from the gel column. The cell described fulfills this criterion by the five separate tubes for transportation of the eluate and by the four cuttings W (Fig. 1). The cuttings are covered by a membrane, that permits passage of the current. This implies that the diameter of the elution chamber in reality equals that of the electrode vessel V2. The field strength and as a consequence the electrophoretic migration rate of a substance are lower in such a cell than in a cell with a diameter equal to that of the gel column.

A pre-requisite for a high resolution is that the gel is uniformly polymerized. The greatest difficulties are encountered at the two horizontal boundaries of the gel. A satisfactory solution of this problem was attained by the procedure utilized in this work (see section a : 2, pp. 20 f.). Further precautions in connection with the polymerization process were the use of carefully deareated solutions, and the flushing of the apparatus with nitrogen (to eliminate the inhibiting effect of oxygen). Worth mentioning are also the effort to polymerize at a constant temperature by the use of both central and outer water-cooling. Ice-water was chosen in order to perform the reaction at a temperature slightly lower than that which would exist within the column during runs. The underlying intention was that when the column was operated, such a gel would not contract, thereby being detached from the surrounding glass walls.

By the introduction of the hydroxylapatite step the final electrophoretic isolation of the two forms of FSH was performed from mixtures of more favourable compositions. This is evident from Figs. 10 (p. 45) and 11 (p. 46) where the two inserts illustrate typical electrophoretic patterns obtained without previous hydroxylapatite chromatography. Both inserts show the presence of inactive components of a high relative concentration

and with mobilities close to those of pituitary and urinary FSH, respectively As both the boundary anomalies and the diffusion are concentration dependent, this implies increased difficulties in preparing the hormones free of contaminants. As far as the pituitary material is concerned the hydroxylapatite step was so effective that the amount of impurities to be eliminated by electrophoresis became relatively small (Fig. 10). In the urinary material on the other hand there remained a more pronounced amount of contaminants which was electrophoretically removed (Fig. 11).

V:2. *Hormone Assay*

The precious material in combination with the small amounts to be assayed made the preparation of the samples for the different tests a very tedious task, involving operations with microcuvettes and constriction pipettes.

The bovine albumin was added in order to stabilize the very diluted hormone samples; concentrations down to 0.03 μg per ml were utilized for the bio-assays and these samples had to be kept for periods of several days. Striking decrease in potency was in fact observed in the absence of albumin, if the hormone concentration was less than 20 μg per ml.

As to the choice of bio-assay methods for FSH the ovarian augmentation method [20] (in rats or mice) is practically the only one now in use. For assaying LH activity the two most commonly used methods are the ovarian ascorbic acid depletion (OAAD) assay [65] in intact female rats and the ventral prostate weight increase method in hypophysectomized male rats (the VPW assay) [66]. The seminal vesicle test [22] used in this study gave valid assays with HCG as reference preparation and HCG gave in its turn valid results against the 2nd IRP. With the OAAD and the VPW methods, valid assays have been reported using ovine preparations (NIH-LH) and the 2nd IRP as reference preparations [26, 67]. With regard to specificity it is therefore no verified evidence to give the preference to any of the three methods. It should be emphasized in this connection that in order to facilitate valid comparisons between different laboratories all preparations should be assayed by the same methods and by use of the same standards. Furthermore standards should, if possible, originate from the same source as the material being assayed.

V:3. *Chemical Characterization of FSH*

A. Amino Acid Analysis

By the method described the calculation of the molecular weight of a protein (or protein moiety of a conjugated molecule) is based upon the analysis

values obtained for a large number of the amino acids occurring in the molecule.

The method suggested is obviously more reliable than basing the calculation on the value of only one of the amino acids occurring in the protein, under the assumption that this value corresponds to a certain integral number of residues. In this latter procedure which is sometimes used the error in the analysis value of the selected amino acid is equalized to zero. This cannot be considered as justified and may result in errors, when calculating the residual numbers of the other amino acids. Furthermore, if such a protein contained a small amount of a contaminant rich in the amino acid selected as a reference there could be large errors in the proposed composition and molecular weight. Such a contamination would be of a much less importance when the calculations are based upon averaged values of several acids.

Another technique to calculate molecular weights of proteins from their amino acid compositions is described by Nyman and Lindskog [68]. These authors studied the sum of the deviations from integral values for all the different amino acids as a function of the molecular weight. Thereby it was neglected that, for amino acids occurring in large amounts, the nearest integral number is not necessarily the most probable. Furthermore, the sum of the deviations as dependable variable is not a good choice, the standard deviation being the relevant parameter. Finally, according to the description given, an accurate knowledge of the amount of protein analyzed is required.

Dealing with pituitary FSH the probabilities of five different compositions were investigated; the lowest value of the coefficient of variation ($s/\bar{k}$) determined the ultimate choice. It was also attempted to investigate if the five values of $s/\bar{k}$ were significantly different, by using the expression

$$\frac{s}{\bar{k}} \sqrt{\frac{1}{2m}\left(1 + 2\left(\frac{s}{\bar{k}}\right)^2\right)}$$

for the standard deviation [69] of $s/\bar{k}$. The values of $s/\bar{k}$ were all small (according to the principle for their selection) and $(s/\bar{k})^2$ was negligible in comparison with 1. It follows that the size of the error in $s/\bar{k}$ is determined by the number (m) of the different amino acids included in the calculation. In the case discussed this number was equal to 9, providing a constant error in $s/\bar{k}$ of about 24 %. Accordingly the above expression for the standard deviation is not applicable to differentiate significantly between the $s/\bar{k}$-values.

The averaged values used for calculating the composition were not always based upon the same number of analyses. This is not strictly correct, as

a value based upon a greater number of determinations is impaired with a smaller error than a value from a lower number of analyses. As, however, the analysis error is not exactly known for any of the amino acids determined (the error of 3 % being an average value), this treatment of the data was considered justified.

The aim with the procedure described was to utilize amino acid analysis data for an independent calculation of molecular weights. (The possibilities to use an electronic computer for the calculations have not yet been explored.) The analysis data for FSH (section A, pp. 50 ff.) gave in combination with the data from the carbohydrate determinations (section D, p. 53) a molecular weight for pituitary FSH of 43,300 and for urinary FSH 27,700. These values are in very good agreement with those independently determined by sedimentation equilibrium centrifugations: pituitary FSH, 41,000; urinary FSH, 26,700–29,400 (see section B, pp. 55 ff.).

B. Estimation of Tryptophan

The general uncertainties involved in accounting for protein absorption as the sum of the contributions from the occurring residues of tyrosine, tryptophan and cystine has been discussed by Wetlaufer [35]. The main difficulty is probably that too little is known about the difference in absorbtivity which seems to exist between the free form of an amino acid and its residue, as it occurs in a protein.

C. Carbohydrate Analysis

Sialic acid has been reported to be a necessary requirement for the biological activity of FSH [72] (see also ref. 77). Pituitary FSH (Table X, p. 53) contained about twice as much sialic acid as did urinary FSH. This fact may contribute to the observed difference in potency between the two forms of FSH.

V:4. *Physical Characterization of FSH*

A. Sedimentation Constant

The values of the sedimentation coefficients are in good agreement with those reported earlier [16, 17, 64] for material prepared by the methods described in sections A:1–A:3 (pp. 15 ff.). Considering the well-documented experience of molecular sieve chromatography acting as an efficient tool for the preparation of fractions of equally sized molecules, this finding is by no means unexpected. It provides, however, further support for the data (Table XI, p. 54) being reliable.

B. Molecular Weight

Preparations of pituitary FSH according to sections A:1–A:3 (pp. 15 ff.) and also material further purified according to section A:5 (pp. 18 ff.) have been subjected to sedimentation equilibrium runs in the ultracentrifuge under the conditions used in the present study. The plots of data were all similar to that shown in Fig. 15 (p. 56); all being slightly concave upward near the bottom of the cell. This repeated feature is suggestive for a tendency of pituitary FSH preparations to aggregate at increased concentration levels.

C. Free Zone Electrophoresis

The mobility values of pituitary and urinary FSH are nearly identical with those reported in connection with the earlier isolation procedure [16, 17, 64] (material prepared according to sections A:1, A:2, A:3 and A:5 (pp. 15 ff. and pp. 18 ff.). This is a good criterion of the reproducibility of the different separation steps involved, especially as other batches of starting material were employed.

D. Ultraviolet Absorption Spectrum

The time-dependence of the spectra in the alkaline medium was indicative of two different processes occurring in the hormone solutions; a rather slow exposure and ionization of absorbing groups and a simultaneous denaturation of the hormones (as visualized by a high relative increase in absorptivities at wavelengths greater than 330 mμ). To correct unequivocally the high non-specific absorption present in these cases was difficult. With linear extrapolation, as suggested here, the correction at 280 mμ was about 40 % of the extinction value at this wavelength (calculated on the values obtained 1–2 hrs after the preparation of the solutions). With logarithmic extrapolation the above correction would be about 50 % of the measured value. Considering the continuous denaturation of FSH and the uncertainty in the correction factors, no attempts were made to calculate the content of tryptophan from the spectra measured in alkali, which is a commonly used method [35].

V:5. *Protein Determination*

The direct reason for determining protein concentration and protein amounts from spectrophotometric data was the avoidance of lyophilization throughout the fractionation procedure. The active fractions were kept in solution and spectrophotometry then became the natural method of choice.

The measurements were performed at 280 mμ as the absorbance of most proteins is maximal close to this wavelength. An error in the wavelength setting therefore resulted in a minimized error in the readings. The value for $A^{1\%}_{1\,cm}$, at 280 mμ, was chosen equal to 10, as this represents an approximate average of the values reported for many different proteins. The spreading for the individual proteins around this value is, however, considerable and it is fully understood that this procedure involves an approximation. The only figure reported for a purified FSH is 9.4 for the ovine hormone [73]. The value for clinical grade FSH (section a:1, pp. 32 ff.) was close to 10 and this was also the case for the active material obtained by further purification on DEAE-cellulose. These values are, however, uncertain as no correction for moisture was made.

Insertion in the expression for Beer's law, of the values for the molecular weights and molar extinction coefficients provided values for $A^{1\%}_{1\,cm}$ equal to 8.2 and 6.3 for pituitary and urinary FSH, respectively. As the values were based on several experimentally determined parameters (see sections A (pp. 50 ff.), B (p. 52), and D (p. 53) they are probably not very precise. As the corresponding values for all the different pituitary and urinary FSH fractions obtained throughout the purification procedure were unknown, it was considered as convenient to persist in using the value of 10 also for the pure hormones. The consequence would be an overestimate of their potencies accompanied by a corresponding underestimate of the yields. The product of potency and yield, i.e. the number of activity units recovered, is independent of the value for $A^{1\%}_{1\,cm}$. These recoveries of pituitary and urinary FSH are given in Tables IV and V, and for permitting a direct comparison, recovered activities after the different steps of purification are also recorded.

It should be emphasized that these recoveries are in each case the recoveries utilized for the continued purification and do not represent the *total* recoveries provided by each of the different separation methods. The explanation is that the primary aim to isolate FSH in pure form has necessitated a very restricted pooling of active material.

In this connection some aspects of the problem of concentrating protein solutions should be considered. The primary reason for avoiding lyophilization or precipitation with organic solvents was the risk for the proteins to undergo polymerization or other conformational changes, which might result in losses of biological activities and provide erroneous values in the ultimate physical characterization.

A recent example of the disadvantages associated with freeze-drying is given by Hanson *et al.* [70]. These workers showed that non-lyophilized human GH prepared according to Roos *et al.* [10] behaved as a single com-

ponent in thin-layer chromatography on Sephadex G-200, although the lyophilized hormone consisted of three different components. That FSH activity is partly lost on lyophilization has been reported by Parlow *et al.* [71] (for material with a potency of about 2000 i.u. per mg) and by Amir *et al.* [72].

In this study only those FSH preparations were routinely lyophilized, which were intended for therapeutic use (potency about 30 i.u. per mg). The lyophilization was performed after dialysis of the preparations against 0.001 *M* NH_4HCO_3. No significant decrease in potency was established after this procedure (see section a:1, pp. 32 ff.). This was also the case at lyophilization of a few preparations further purified by DEAE-cellulose chromatography (potency about 300 i.u. per mg). Lack of material has prevented the performance of reliable studies of the stability of the more highly purified fractions.

Concentrating protein solutions by ultrafiltration enables (1) the procedure to be terminated at any desired volume and (2) the sample (still in the same dialysis tube) to be equilibrated with the buffer which will be employed in a subsequent fractionation procedure. In this way ultrafiltration provides the sample in the desired buffer and in an appropriate volume with a minimum of manipulations. With the procedure according to von Hofsten and Falkbring [11] the recoveries were consistently about 90%. With the collodion sacs, employed for concentrating smaller amounts of material (sometimes to volumes of about 0.5 ml), the losses were in the range of 10–20%.

To determine yields and activities of FSH on a spectrophotometric basis has been subjected to criticism [27]. However, Donini *et al.* [27] seem to have underestimated the difficulties in weighing proteins in an unequivocal way. They state that they have been able to "correlate biological activities to dry weight", ignoring the fact that the term dry weight means the weight after correction for moisture. In addition these workers made a direct comparison between the weight of lyophilized material and the weight of material that had been precipitated by alcohol, washed with alcohol and ether and then air dried. The high probability that samples that have been treated so differently will have quite different water contents was overlooked.

V:6. *Comparison of Some Properties of Pituitary and Urinary FSH*

Some properties of pituitary and urinary FSH are briefly discussed in this section.

The conditions for adsorption-desorption of pituitary FSH using DEAE-cellulose columns and hydroxylapatite columns were also applicable to

Table XII. Some parameters determined for human pituitary and urinary FSH.

$s^0_{20,w}$ S	Mol. wt.	f/f_0	Mobility $cm^2\,sec^{-1}\,volt^{-1}$	ε mol^{-1} 1 cm^{-1}	Residues per mole			Biological FSH activity i.u./mg	Immunological LH activity i.u./mg
					Hexose	Glucosamine	Sialic acid		
itary FSH									
.0	41,000	1.68	$-5.83 \cdot 10^{-5}$	35,500	39	30	8	14,000	18
ıary FSH									
.2	28,000	1.75	$-6.17 \cdot 10^{-5}$	17,400	14	8	2	780	15

the purification of urinary FSH. Preparative chromatography on Sephadex G-100 did not reveal any pronounced difference in the elution volumes for pituitary and urinary FSH. To further investigate the possible existence of a difference the following experiments were performed. A column of Sephadex G-100 (0.85×200 cm) was equilibrated in 0.1 *M* potassium phosphate buffer, pH 7.0, also containing 0.3 *M* NaCl. Five samples were separately chromatographed; in runs 1, 3 and 5 one mg of bovine albumin was applied to the column, in runs 2 and 4 one mg of pituitary and urinary FSH, respectively. The sample volume was 1 ml in each case, the flow rate about 2.5 ml/cm^2/h and fractions of about 0.8 ml were collected. The protein content was measured by spectrophotometry at 280 mμ and the elution volume for the peak tube was in each experiment accurately measured. The three experiments with albumin indicated a high reproducibility ($Ve = 56.3 \pm 0.2$ ml). The elution volumes of pituitary and urinary FSH were 59.6 ml, and 59.1 ml, respectively; a difference too small to be significant as only one experiment was performed with each preparation and the fraction volumes were 0.8 ml. In polyacrylamide gel electrophoresis the migration velocities were approximately the same for the two forms of FSH.

In Table XII some of the parameters determined for pituitary and urinary FSH are summarized. They are all given earlier in the text except for the frictional ratio, f/f_0, which was calculated from the ultracentrifugation data [42]. The values are similar and comparatively high, suggesting elongated molecules. As, however, f/f_0 is a composite function of molecular shape and hydration no distinct conclusions as to the shape should be drawn.

The data summarized this far is not inconsistent with the view that urinary FSH represents a main fragment of pituitary FSH, retaining some structure responsible for the biological activity. The data from the amino acid analyses are, however, not compatible with such a postulate. It is therefore suggested that formation of urinary FSH from pituitary FSH has involved

both substraction and addition of glycopeptides and/or peptides. The possible occurrence of altered gonadotropin molecules has been discussed by Ellis [74], with reference to the reduction of the disulfide bonds of insulin by hepatic glutathione insulin-transhydrogenase [75], giving rise to free A and B chains. A similar degradation of pituitary FSH in association with aggregation to some circulating or excreted substance might explain the differences in amino acid composition between pituitary and urinary FSH. It is worth noting that evidence has been presented for the existence in human plasma of albumin as a mixed disulfide compound with cysteine or glutathione [76].

V:7. *Work by Other Investigators*

Work on the extraction and purification of pituitary and urinary FSH [77, 78] and of pituitary LH [78] has recently been treated in review articles. In the following, papers of a later appearance dealing with these hormones, will be discussed. The results obtained will be compared, whenever possible, with those presented earlier in this thesis.

A. Pituitary Material

The serial extraction procedure of Wilhelmi [79] for acetone-preserved human pituitaries provided two FSH-rich fractions (Ab and Ac). Highly significant amounts of LH activity were also present in these two fractions. Reichert and Parlow [80] have utilized fraction Ab for the partial purification and separation of FSH and LH by chromatography on DEAE-cellulose. The FSH and LH potencies of this starting material were 4.6 U[1] per mg (120 i.u. per mg) and 0.36 U[2] per mg (550 i.u. per mg), respectively. An average yield of fraction Ab from many batches was 3.8 mg per g dry weight of pituitary or 300 mg per 1000 pituitaries (using a value of 80 mg as an average for the dry weight of each pituitary [81]). The chromatographic procedure provided a principal FSH-containing fraction with a potency of 29 U per mg (770 i.u. per mg). From the data given the yield from 1000 pituitaries can be calculated to be 32 mg, containing about 25,000 i.u. of FSH activity. The active fraction was further purified by ion exchange chromatography and gel filtration [82]. Bio-assays of the content in individual test tubes indicated the presence in one single tube of material with an FSH potency of 94 U per mg (2500 i.u. per mg). Each of the two adjacent fractions

[1] 1 U = the activity in 1 mg of NIH-FSH-S1 – 26.6 i.u. [27].
[2] 1 U = the activity in 1 mg of NIH-LH-S1 = 1538 i.u. [27].

was about half as potent. No yield was given or could be calculated from the data presented.

The principal LH-containing fraction prepared by Reichert and Parlow [80] had an activity of 0.90 U per mg (1400 i.u. per mg). The yield which can be calculated from 1000 pituitaries is 60 mg, containing 84,000 i.u. of LH activity. Further purification of this fraction by gel filtration [82] or of similar fractions by chromatography on Amberlite IRC-50 [83] were reported to give LH preparations with potencies of 2–3 U per mg (3100–4600 i.u. per mg). From the data provided no yields in terms of the starting material can be calculated.

Parlow *et al.* [71] described a multiple chromatographic system for the separation and purification of FSH, LH and TSH (thyrotropic hormone). The starting material was fraction Ab of Wilhelmi [79] and also in this paper [71] the yields were not explicitly given neither could they be calculated from the data provided. An FSH fraction with a potency of 75 U per mg (2000 i.u. per mg) was prepared. Its immediate precursor was reported to have a potency of 17 U per mg (450 i.u. per mg), retaining 74 % of the total FSH activity of fraction Ab. Referring to the value of 300 mg per 1000 pituitaries as the yield of fraction Ab the yield of the above precursor would be 48 mg from this number of glands. The principal LH fraction was of a potency of 2.8 U per mg (4300 i.u. per mg). The activity recovered in this fraction was reported as 19 %, corresponding to 11 mg per 1000 pituitaries.

Parlow *et al.* [81] have recently simplified the extraction procedure of Wilhelmi for acetone-preserved pituitary glands. The main part of FSH was found in one extract (fraction A), in a yield of 1700 mg per 1000 glands and at a potency of 2.1 U per mg (56 i.u. per mg). The LH activity was distributed between fraction A and another fraction (designated Bb4N). The LH potency of fraction A was 0.13 U per mg (200 i.u. per mg). With fraction A as a starting material DEAE-cellulose chromatography was utilized for the partial separation of FSH and LH. The FSH potency was reported as 5.20 U per mg (138 i.u. per mg) and the LH potency as 0.57 U per mg (877 i.u. per mg). From the data provided the yield of the two hormone preparations can be calculated to be 625 mg and 170 mg, respectively, from 1000 pituitaries. Consequently 86,000 i.u. of FSH activity and 150,000 i.u. of LH activity are recovered from 1000 pituitaries.

According to Parlow *et al.* [81], the activity recoveries of the gonadotropins from acetone preserved glands were substantially the same as those from frozen glands, when utilizing Wilhelmi's modified procedure.

Elrick *et al.* [84] have described a procedure which resulted in the concentration of FSH, LH and TSH into one fraction. The pituitaries were

preserved in acetone containing mercaptoethanol (0.1 %) and acetic acid (1 %). This solvent was also used to extract the glands and prepare a pituitary powder. The hormone concentrate was obtained by extraction of the powder at pH 5.5 in the presence of mercaptoethanol (0.1 %). Based upon the data provided the yield from 1000 pituitaries can be calculated to be 6.3 g. The FSH potency of the fraction was 0.28 U per mg (7.4 i.u. per mg) and the LH potency 0.11 U per mg (170 i.u. per mg).

Another procedure for the separation and partial purification of pituitary hormones from acetone-dried pituitaries is that of Hartree [85]. A gonadotropin concentrate was prepared mainly as described by Koenig and King [86] in a yield of 2.2 g per 1000 glands and with an FSH potency of 1.1 U[1] per mg (28 i.u. per mg). Partial separation of FSH and LH was obtained by chromatography on CM-cellulose, leaving most of the LH activity initially adsorbed to the cellulose. FSH was unretarded and the yield from 1000 glands was reported as 1.1 g with a potency of 3.4 U per mg (88 i.u. per mg). The FSH fraction has been further purified by chromatography on DEAE-cellulose and calcium phosphate followed by partition between urea-ammonium sulphate-ethylene glycol monoethyl ether [26]. The FSH potency was reported as 1036 i.u. per mg. No yield was given and subsequent starch gel electrophoresis indicated four main components.

Desorption of the LH activity from the CM-cellulose column was followed by chromatography on DEAE-cellulose essentially as described by Hartree *et al.* [87]. An LH fraction (DEAE-1) weighing 70 mg and having a potency of 3.6 U[2] per mg (5400 i.u. per mg) was obtained from 1000 pituitaries. To further increase the LH potency, chromatography on Amberlite IRC-50 was subsequently applied. One preparation reached a potency of 5.3 times that of NIH-LH-S3 and another was 4.8 times as potent as NIH-LH-S1 (equivalent to 7200 i.u. of LH activity per mg). No yields of these preparations were given.

A recent paper dealing with human FSH is that by Amir *et al.* [72]. The starting material was prepared from acetone-dried pituitaries according to Butt *et al.* [88]. Serial passage through columns with CM-cellulose, calcium phosphate, Sephadex G-100 and DEAE-Sephadex was performed. No yields whatsoever were reported and the assay data were not expressed on a basis, permitting comparison with the data of others. The paper also contains undocumented conclusions as regards the presence of free SH-groups in FSH and their role for maintaining the biological activity of the hormone. It should be recalled that the amino acid analysis data presented

[1] 1 U = the activity in 1 mg of NIH-FSH-S1 = 25.7 i.u. [26].

[2] 1 U = the activity in 1 mg of NIH-LH-S1 = 1500 i.u. [26].

Table XIII. Potency, weight yield and recovered activity of different human pituitary FSH preparations.

No.	Reference	Designation of fraction	FSH potency, i.u./mg	Weight yield, mg/1000 pituitaries	Recovered activity, i.u./1000 pituitaries
1	80, 81	Ab	120	300	36,000
2	81	A	56	1700	95,000
3	84	Extract 1	7.4	6300	47,000
4	85	Glycoprotein fraction	28	2200	62,000
5	Table IV		29	17,000[a]	490,000
6	80, 81	F-1-4	770	32	25,000
7	71, 81	R-470-4	140	625	88,000
8	85	CM-1	88	1100	97,000
9	Table IV		300	620[a]	190,000
10	82	Tube 21	2500	—	—
11	71, 81	18-70A1 18-70A2	450	48	22,000
12	Table IV		3300	40[a]	130,000
13	Table IV		14,000	1.8[a]	25,000

[a] From Table IV, using a weight of 0.62 g for one frozen pituitary gland.

in this thesis (pp. 50 ff.) did not show the presence in FSH of any free SH-groups.

In Table XIII some of the yields and potencies of different FSH preparations are summarized. Data from Table IV (p. 42) is also included for comparison. Fractions 1–4 were prepared only by extraction and precipitation procedures as was fraction 5. It can be seen that the recovered activity in fraction 5 was significantly higher than the recovery in fractions 1–4. Fractions 6–8 were obtained by further purification utilizing ion-exchange column chromatography. Fraction 9 was prepared by comparable procedures. The activity recovered in this fraction was at least twice as high as the recovery in any of fractions 6–8. When fractions 10 and 11 are compared with fraction 12, the latter is seen to be the more active. Fraction 13 is the most potent pituitary FSH prepared in this study.

The LH preparations discussed above were all assayed by the OAAD method. No factor is available to correlate data obtained by this method with those from the seminal vesicle test used in the present work. A valid comparison between the different LH preparations is therefore not possible. However, it is noteworthy that the procedure of Parlow *et al.* [71] resulted

in a 5.4-fold increase in LH potency over that of the original precursor. Using the procedure in section B (pp. 46 ff.), the final product was about 200 times as potent as the starting material.

No chemical or physical parameters of highly purified human pituitary FSH are available for a comparison with those reported in this study.

B. Urinary Material

Reichert and Parlow [89] have fractionated gonadotropin concentrates prepared from postmenopausal urine and from eunuch urine. The concentrates were prepared essentially as described by Albert *et al.* [90] and fractionated by DEAE-cellulose column chromatography under conditions earlier used [80] for the purification of pituitary concentrates.

The postmenopausal urine concentrate had an FSH potency of 0.67 U[1] per mg (18 i.u. per mg) and an LH potency of 0.0086 U[2] per mg (13 i.u. per mg). The yield per liter of urine was not given in explicit form, but from the data provided 0.7 mg seems to be a fair estimate. DEAE-cellulose chromatography resulted in an unadsorbed fraction with an LH potency of 0.022 U per mg (34 i.u. per mg). In the principal FSH fraction the potency, as estimated according to Steelman and Pohley [20], was 1.90 U per mg (50.5 i.u. per mg); 77 % of the applied FSH activity being recovered. The LH activity of this fraction was 0.0035 U per mg (5.4 i.u. per mg).

Fractionation under the same conditions of an eunuch urine concentrate (FSH potency 0.46 U per mg; LH potency 0.0021 U per mg) gave a very similar elution pattern. The relevant data for the principal FSH fraction were: FSH potency, 4.1 U per mg (109 i.u. per mg); LH potency, 0.0021 U per mg (3.2 i.u. per mg). 41 % of the FSH activity of the starting material was recovered in this fraction.

Donini *et al.* [91] have prepared a gonadotropin concentrate from postmenopausal urine by using essentially the procedure of Albert *et al.* [92] followed by permutit column chromatography. An average yield from four batches was 0.51 mg per liter of urine. Fractionation of the concentrate was achieved by gel filtration on Sephadex G-200 [93]. A fraction with an FSH potency of 88.7 i.u. per mg and an LH potency of 57 i.u. per mg (VPW) was obtained. This fraction contained 69 % of the total FSH activity applied.

In a recent publication Donini *et al.* [27] reported a comparative study in order to find the best methods for the further separation and purification of FSH and LH. The starting material [91] had an FSH potency of 49.1 i.u. per mg and an LH potency of 33.6 i.u. per mg. Four different procedures

[1] See footnote 1, p. 78.

[2] See footnote 2, p. 78.

were compared; (A) column electrophoresis at pH 8.6 in cellulose powder; (B) column chromatography on DEAE-cellulose essentially according to Reichert and Parlow [80]; (C) pooled active material from procedure (A) was fractionated according to procedure (B); (D) column chromatography on DEAE-cellulose followed by two consecutive runs on Sephadex G-100 under the conditions described by Roos and Gemzell [64].

The most active fraction prepared by procedure (A) had an FSH potency of 154 i.u. per mg and an LH potency of 15.3 i.u. per mg (OAAD). The yield from 1 g of starting material was 56 mg accounting for 17 % of the total FSH activity applied. More representative for the potentialities of the electrophoretic step are probably the figures obtained from pooled material, used as starting material in the DEAE-cellulose step of procedure (C). These are as follows: 85.2 i.u. of FSH activity per mg, 21.6 i.u. of LH activity per mg, 358 mg per g of starting material, 62 % of total FSH activity.

Procedure (B) resulted in one principal FSH-containing fraction, the LH activity being evenly distributed over the main part of the chromatogram. An average yield of the FSH fraction from two runs was 286 mg per g of starting material. Average values of the FSH and LH potencies were 131.8 i.u. per mg and 24.0 i.u. per mg, respectively. The recovery in the principal fraction of FSH activity was 77 %.

The chromatographic step of procedure (C) gave a similar partition of the hormone activities as that obtained by procedure (B). The relevant data for the principal FSH fraction were: 189.0 i.u. of FSH activity per mg, 13.6 i.u. of LH activity per mg. A yield of 91 mg per g of starting material (the gonadotropin concentrate) and a recovery of 35 % of the FSH activity can be calculated from the data provided.

The results obtained by procedure (D) confirm those earlier reported by Roos and Gemzell [17, 64]. The unadsorbed material from the DEAE-cellulose column accounted for about 50 % of the total LH activity applied and in the subsequently eluted fraction 85 % of the FSH activity was recovered. The yield of this latter fraction was 456 mg from each g of the starting material. The FSH and LH potencies were 91.6 and 18.4 i.u. per mg, respectively. Following the two runs on Sephadex G-100 the FSH potency increased to 384.4 i.u. per mg and the LH potency decreased to 5.6 i.u. per mg. The yield of the fraction having these potencies can be calculated to be 29 mg per g of the gonadotropin concentrate, retaining 23 % of the original FSH activity.

Assuming the yield of 0.51 mg of gonadotropin concentrate per liter of urine [91], the above yield is $15 \cdot 10^{-3}$ mg per liter of urine.

The cross-reaction between antibodies against HCG and urinary LH, originally reported by Wide *et al.* [94], has been utilized by Donini *et al.*

Table XIV. Potency and recovered activity of different human urinary FSH preparations.

No.	Reference	Designation of fraction	FSH potency i.u./mg	Recovered activity %
1	89	C-PM-5-A precursor	18	
2	27	P-28E54C	49.1	
3	Table V		8.6	
4	89	D-PM-2-3	51	77
5	27	Exp. no. 2, fraction 4	132	77
6	27	Exp. no. 4 a, fraction B	91.6	85
7	Table V		120	60
8	27	Exp. no. 4 b, fraction 5	384	23
9	Table V		380	38
10	Table V		780	7.7

[95] as an approach to the preparation of biologically pure urinary FSH. The starting material was the same urinary concentrate as mentioned above [91]. Incubation with anti-HCG serum was followed by DEAE-cellulose chromatography under the same conditions as in procedure (B) [27]. The data for the principal FSH fraction were as follows: FSH potency, 148.3 i.u. per mg; LH potency, 2.4 i.u. per mg; yield per g, 278 mg; and recovery of total FSH activity, 84 %.

The method has also been applied starting with material prepared according to procedure (D) [27]. A principal FSH fraction was obtained with a potency of 329.7 i.u. per mg. Its LH potency was reported to be 0.75 i.u. per mg. The full significance of these potencies are difficult to evaluate as no activities of the starting material were provided. The high degree of biological purity of the FSH fraction, suggested by its low LH content, was not paralleled by a similar degree of chemical purity. The fraction was reported to contain both contaminants originating from the starting material and from the rabbit serum added for incubation prior to chromatography. Finally, the report by Robyn *et al.* [96] of antibodies against HCG which cross-react with urinary LH as well as with urinary FSH, leaves the procedure by Donini *et al.* [95] open for further experiments and discussion.

Some of the data for the different FSH preparations obtained from postmenopausal urine are summarized in Table XIV. Data from Table V (p. 44) is also included.

It is difficult to make an unequivocal comparison of the potencies and yields of preparations from the different laboratories. In the present study

as well as in the work by Reichert and Parlow [89] the starting material (preparations 3 and 1, respectively, in Table XIV) was urinary concentrates prepared by others. In contrast, Donini *et al.* [91] started from unconcentrated collections of urine. Thus it is not relevant to relate recoveries to volume of urine in every case, and other methods of comparison must be sought. This point should be borne in mind when considering the urinary concentrate prepared by Donini *et al.* [27] (preparation 2, Table XIV), using methods similar to those employed for fractions 1 and 3 and which had an FSH potency 2.7 and 5.4 times higher than the potencies of these fractions.

More reliable parameters for comparison would be the potencies and the percentage activity recovered following the use of similar separation methods. Data of this type is provided in Table XIV. Fractions 4–7 were prepared by DEAE-cellulose chromatography. Fractions 8 and 9 were obtained by further purification utilizing molecular sieve chromatography. The potencies of these two fractions were very similar; the recovered activity in fraction 11 was, however, about 60 per cent higher. Fraction 10 was the most potent urinary FSH prepared in this study.

No published data is available to enable a comparison of the chemical and physical parameters of urinary FSH.

VI. CLINICAL APPLICATION

VI:1. Treatment of Female Sterility

The clinical use of pituitary gonadotropins in women has recently been reviewed [97, 98]. In Uppsala, during a 4 year period, Professor Carl Gemzell (Akademiska sjukhuset) has utilized preparations of human pituitary FSH for the treatment of about 150 women with primary or secondary amenorrhea. The original preparation method of Gemzell *et al.* [1] from lyophilized glands has been replaced by the procedure described in section a:1 (pp. 15 f.). In a few cases [99] material from acetone-preserved glands (section a:2, p. 16) was employed.

In general each patient receives daily injections of 100 i.u. of FSH for 6–10 days, followed by 3 daily doses of HCG, each containing 3000 I.U. A total of 64 conceptions have followed treatment.

VI:2. Treatment of Male Sterility

Professor Carl Gemzell and Dr Berndt Kjessler (Akademiska sjukhuset, Uppsala) have used FSH preparations (according to section a:1) for treating male sterility. After treatment, four men with aspermia produced sperms and two of them reached fully fertile levels of sperm production; one of the latter later became a father to a normal child ([100] and Kjessler, personal communication, 1967).

The treatment schedule employed was simultaneous injections of 150 i.u. of FSH and 500 I.U. of HCG twice a week for 12 weeks.

VI:3. Treatment of Hypo-pituitary Dwarfism

GH prepared according to Roos *et al.* [10] (in a yield of about 3 mg per gland and with a biological potency of 1–2 USP units[1] per mg) is utilized in several cases diagnosed as hypopituitary dwarfs.

At present, Dr Otto Westphal (Akademiska sjukhuset, Uppsala) has ten patients: of these one has been treated for 4 years and three for 3 years, the others for shorter periods of time. On average the four patients treated for 3–4 years increased in length by 6–7 cm a year (during the first year of treatment the increase was about 10 cm). A recent examination of sera

[1] 1 unit = the activity in 1 mg USP-GH (bovine).

from the ten patients (by radioimmunological technique [24]) did not reveal any antibodies against GH. This is an important finding as it has been reported [101] that human GH prepared under other conditions [2] may elicit the formation of antibodies in such high titres that the effect of GH on growth and metabolism is neutralized. The absence of GH antibodies in the ten sera are indicative of a very low content of denaturated GH molecules in the preparation administered.

Dr C. G. Bergstrand (Kronprinsessan Lovisas Barnsjukhus, Stockholm) has treated five patients with GH, of which three are still under treatment. One of the patients, a girl who is now 7 years of age, has increased in height from 85 cm to 119 cm during three years of treatment.

Dr Björn Sjögren (Sahlgrenska sjukhuset, Göteborg) has two patients, both treated for about one year. One of them, a 19 year old girl, increased her height with 10.8 cm. Her average increase during a 10 year period prior to treatment was 2.9 cm per year.

The doses of GH used in these clinical studies were 2 mg twice a week (Westphal and Bergstrand) or three times a week (Sjögren).

Finally, Seip and Trygstad [102] have reported upon the treatment of 12 patients with GH prepared by them according to Roos *et al.* [10]. The periods of treatment were between 6–41 months and none of the patients became unresponsive to the hormone. Examination of sera from two siblings, treated for 3.5 years, did not show the presence of antibodies against GH. This observation is thus in agreement with the data of Westphal.

VII. SUMMARY

1. Human FSH (a glycoprotein) has been isolated from whole frozen pituitaries and from a postmenopausal urinary concentrate. The procedure for the preparation of pituitary FSH involved extraction of the homogenized glands, ammonium sulphate fraction, DEAE-cellulose chromatography, molecular sieve chromatography on Sephadex G-100, hydroxylapatite chromatography and polyacrylamide gel electrophoresis. The last four fractionation methods were employed also for the isolation of urinary FSH.

An apparatus for preparative polyacrylamide gel electrophoresis was constructed and utilized for the isolation of FSH.

2. The yield of human pituitary FSH was about 0.3 mg per 100 g of pituitary tissue or about 2 μg per pituitary, with a biological potency of about 14,000 i.u. of FSH activity per mg. The yield of urinary FSH was about 0.7 mg per g of the urinary concentrate or about 1 μg per liter of postmenopausal urine. The biological potency of urinary FSH was about 780 i.u. of FSH activity per mg. These potencies are the highest which have been reported for human pituitary and urinary FSH.

3. In the ultracentrifuge both forms of FSH sedimented as a single component. The data were compatible with sedimentation constants of 3.0 S and 2.2 S for pituitary and urinary FSH, respectively. Molecular weight determinations by equilibrium centrifugation gave values of 41,000 for pituitary FSH and approximately 28,000 for urinary FSH. Both hormone preparations were homogeneous when examined by free zone electrophoresis. The mobilities at pH 8.7 and 0°C were $-5.83 \cdot 10^{-5}$ cm^2 sec^{-1} volt^{-1} and $-6.17 \cdot 10^{-5}$ cm^2 sec^{-1} volt^{-1} for pituitary and urinary FSH, respectively.

4. Amino acid analysis indicated that pituitary and urinary FSH contained 254 and 208 amino acid residues, respectively. A method was suggested for the calculation of molecular weights from amino acid analysis data. Values of 29,814 and 23,600 were obtained for the protein moietes of pituitary and urinary FSH, respectively. The molecular weights of the carbohydrate moieties were found to be 13,487 and 4142, respectively. From this data the molecular weights of pituitary and urinary FSH were calculated to be 43,300 and 27,700, respectively, in good agreement with the values independently determined by ultracentrifugations.

The molar extinction coefficient for pituitary FSH at 280 mμ and neutral pH was 35,500 mol^{-1} 1 cm^{-1} and for urinary FSH the value was 17,400 mol^{-1} 1 cm^{-1}. The partial specific volumes were calculated to be 0.69 g^{-1} cm^{-3} for both preparations.

5. Gel diffusion analysis using antiserum to a pituitary FSH preparation showed a reaction of partial identity between pituitary and urinary FSH. The single precipitin line given by pituitary FSH was further supporting evidence of its homogeneity.

6. The amount of material used in the study of the properties of the two forms of FSH was in each case only 6–7 mg.

7. A brief description of the purification of human pituitary LH was given. The procedure made use of chromatography on DEAE-cellulose, Sephadex G-100 and SE-Sephadex C-50. The yield of this preparation was about 1.3 mg per 100 g of pituitary tissue, with a biological potency of about 14,000 i.u. of LH activity per mg. By radioimmunological assays the potency was about 18,000 i.u. of LH activity per mg. In terms of this latter value, the content of LH in the most active pituitary and urinary FSH preparations was estimated to correspond to maximally 0.1 per cent by weight.

8. Some clinical results of the application of a pituitary FSH concentrate in the treatment of male and female sterility were given. Also included were some clinical results of the treatment of hypo-pituitary dwarfism with human pituitary growth hormone (prepared according to a method described elsewhere).

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